DOES NOT CIRCULATE

THIS PART COMPLETES VOLUME 55 (1953)

DECEMBER 1953, VOLUME 55, No. 5

UNIVERSITY OF MICHIGAN

JAN 9 1954

MEDICAL LIBRARY

THE BIOCHEMICAL JOURNAL

Edited for

THE BIOCHEMICAL SOCIETY

Editorial Board

A. NEUBERGER (*Chairman*) R. K. CALLOW (*Deputy Chairman*)

R. E. DAVIES R. MARKHAM

S. R. ELSDEN N. H. MARTIN

D. HERBERT A. G. OGSTON

W. KLYNE R. L. M. SYNGE

C. LONG W. V. THORPE

M. G. MACFARLANE W. J. WHELAN

T. MANN T. S. WORK

CAMBRIDGE UNIVERSITY PRESS

LONDON: BENTLEY HOUSE, N.W. 1

NEW YORK: 32 EAST 57TH STREET, 22

Price 20s. net

The *Biochemical Journal* is conducted by the **Biochemical Society** and is published by the Cambridge University Press. The *Journal* is issued in multiple volumes per year. Each volume consists of approximately 700 pages, published in parts at intervals of one month.

The Biochemical Society was instituted solely for the advancement of the Science of Biochemistry, and holds meetings at different centres for the communication of original papers, and for the discussion of current problems. Persons interested in Biochemistry are eligible for election. Members whose subscriptions are not in arrears are entitled to receive the *Biochemical Journal* without further payment. Further information may be obtained from the Hon. Secs., Professor R. H. S. Thompson, Department of Chemical Pathology, Medical School, Guy's Hospital, London, S.E. 1, and Professor F. L. Warren, Department of Biochemistry, London Hospital Medical College, Turner Street, London, E. 1, or from the Hon. Treas., F. A. Robinson, Esq., c/o Allen and Hanburys, Ltd., Bethnal Green, London, E.C. 2.

Subscriptions to the *Biochemical Journal*. For non-members of the Biochemical Society the subscription to the *Biochemical Journal* is £3. 10*s*. 0*d*. per volume, payable in advance to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1, to F. A. Robinson, Esq., c/o Allen and Hanburys, Ltd., Bethnal Green, London, E.C. 2, or to any bookseller. In the United States of America the *Journal* may be purchased from the Cambridge University Press American Branch, 32 East 57th Street, New York, 22 (subscription price $12.25 per volume).

Communications respecting the sale of single issues or back numbers of the *Journal*, Volumes 20–52, should be addressed to the Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1. Volumes 1–15 have been reproduced and are available from Messrs William Dawson & Sons, Ltd., 102 Wigmore Street, London, W. 1, at the prices given below.

Claims for the replacement of *Journals* lost in transmission will not be entertained if they are received later than three months after the date of the posting of the *Journal*.

Prices of back numbers of the *Journal*.

Volume 1–15 complete.	£82. 10*s*. bound, £75 unbound
Separate volumes.	£6 each bound, £5. 10*s*. each unbound

Reduced terms for Volumes 1–15 are available to members of the Biochemical Society, who should apply to the Honorary Secretaries.

	In paper cover (except Vol. 38)
Volumes 20–28.	£8 per volume of 6 parts at £1. 12*s*. each if sold separately
Volumes 29–35.	£8 per volume of 12 parts at 16*s*. each if sold separately
Volume 36.	£5 per volume of 12 parts at 10*s*. each if sold separately
Volumes 37 and 40.	£5 per volume of 6 parts at £1 each if sold separately
Volumes 41–43.	£5 per volume of 4 parts at £1. 10*s*. each if sold separately
Volumes 38, 39, 44–51.	£5 per volume of 5 parts at £1. 4*s*. each if sold separately
Volumes 52, 53, 54.	£4 per volume of 4 parts at £1 each if sold separately
Index to Volumes 11–20.	10*s*. net
Index to Volumes 21–30.	15*s*. net
Index to Volumes 31–40.	£1. 15*s*. net
History of the Biochemical Society.	2*s*. 6*d*. net

Binding. Quotations can be given by the publishers for bound copies of back numbers; also for buckram binding cases, and for binding subscribers' sets. (Suspended.)

CONTENTS OF VOLUME 55, No. 5, 1953

(*All rights reserved*)

PAGE

The assimilation of amino acids by bacteria. 18. The incorporation of glutamic acid into the protein fraction of *Staphylococcus aureus*. By E. F. Gale and J. P. Folkes. (With nine figures) 721

The assimilation of amino acids by bacteria. 19. The inhibition of phenylalanine incorporation in *Staphylococcus aureus* by chloramphenicol and *p*-chlorophenylalanine. By E. F. Gale and J. P. Folkes. (With three figures) 730

The reaction of fluorodinitrobenzene with the α- and ε-amino groups of collagen. By J. H. Bowes and J. A. Moss 735

The catalytic effect of molybdate on the hydrolysis of organic phosphates. By H. Weil-Malherbe. . 741

Studies with marked antisera. Quantitative studies with antisera marked with iodine ^{131}isotope and their corresponding red-cell antigens. By J. C. Boursnell, R. R. A. Coombs and V. Rizk. (With fourteen figures) 745

The chemical composition of the soluble and insoluble fractions of the bacterial cell. By W. R. Fernell and H. K. King. (With two figures) 758

The mechanism of inhibition of cholinesterases by organophosphorus compounds. By W. N. Aldridge and A. N. Davison. (With three figures) 763

Physical studies on a soluble protein obtained by the degradation of elastin with urea. By T. J. Bowen. (With three figures) 766

Glycerylphosphorylethanolamine in rat brain. By G. B. Ansell and J. M. Norman 768

The bases of the nucleic acids of some bacterial and animal viruses: the occurrence of 5-hydroxymethylcytosine. By G. R. Wyatt and S. S. Cohen. (With one figure) 774

The synthesis of lactose from glucose in the mammary gland. By O. K. Reiss and J. M. Barry. (With two figures) 783

Alkaline phosphatase of milk. 1. Association of the enzyme with a particulate lipoprotein complex. By R. K. Morton. (With one figure) 786

Alkaline phosphatase of milk. 2. Purification of the enzyme. By R. K. Morton. (With one figure) . 795

Oxidative pathways in a fluorescent *Pseudomonas*. By M. Kogut and E. P. Podoski. (With seven figures) 800

Observations on brain phosphatases. By J. J. Gordon 812

Biosynthesis of choline in the seedling of the chick-pea (*Cicer arietinum*). By K. Ahmad and M. A. Karim 817

Quantitative estimation of ascorbic acid and related substances in biological extracts by separation on a paper chromatogram. By Y.-T. Chen, F. A. Isherwood and L. W. Mapson 821

Separation and estimation of organic acids on paper chromatograms. By F. A. Isherwood and C. S. Hanes. (With three figures) 824

The combination of some vitamin B_{12}-like compounds with sow's milk whey and 'intrinsic factor' concentrates. By M. E. Gregory and E. S. Holdsworth. (With one figure) 830

The pigments in colour phases of the larvae of *Plusia gamma* L. (the Silver-Y moth). By T. W. Goodwin. (With two figures) 834

The fungicidal activity of substituted acetanilides and related compounds. By N. G. Clark and A. F. Hams 839

The deamidation of nicotinamide by bacteria. By D. E. Hughes and D. H. Williamson. (With five figures) 851

The phosphorylation and absorption of sugars in the rat. 1. Hexokinase activity in the intestinal mucosa. By M. P. Hele. (With two figures) 857

PAGE

The phosphorylation and absorption of sugars in the rat. 2. Sugar absorption *in vivo*, and its relationship to the phosphorylation of sugar *in vitro*. By M. P. Hele. (With one figure) 864

A chromatographic study of uroporphyrins from cases of cutaneous, acute and congenital porphyria. By J. Canivet and C. Rimington. (With one figure) 867

The chromatographic separation of free and combined plasma cholesterol. By L. M. H. Kerr and W. S. Bauld. (With one figure) 872

Oxidative fission of pregnanediol glucuronide. By J. K. Norymberski and A. T. Sermin. (With one figure) 876

Active transport of water, sodium, potassium and α-oxoglutarate by kidney-cortex slices. By R. Whittam and R. E. Davies. (With two figures) 880

The enzymic hydrolysis of acid anhydrides. By F. Bergmann, M. Wurzel and E. Shimoni. (With four figures) 888

The possible role of coenzyme A in the biosynthesis of cholesterol in the rat. By G. S. Boyd. (With one figure) 892

Proceedings of the Biochemical Society

PAGE

Oxidative phosphorylation in insect sarcosomes. By S. E. Lewis and E. C. Slater xxvii

The effect of dinitrophenol on insect sarcosomes. By E. C. Slater and S. E. Lewis xxvii

A study of the complexes of some simple peptides with zinc and cadmium ions in aqueous solution. By D. J. Perkins xxviii

The molecular rotations of triterpenoid alcohols and their derivatives. By W. Klyne and W. M. Stokes xxviii

Synthesis and oxidation of citric acid in mammary gland homogenates. By C. Terner xxix

The enzyme complement of bean root cells. By E. Robinson xxx

The separation of α-keto acids in blood and urine by paper chromatography. By M. J. H. Smith and K. W. Taylor xxx

Effect of inhibitors on the metabolism of *Microsporum*. By A. J. E. Barlow, F. W. Chattaway and C. C. Thompson xxxi

The site of conversion of carotene injected into rats. By J. G. Bieri and C. J. Pollard xxxi

Tryptophan-tryptophanase reaction. Consideration of the nature of the enzyme-coenzyme-substrate complex. By H. Gooder and F. C. Happold xxxii

A possible model of the tryptophan-tryptophanase action. By J. W. Baker xxxii

The fate of intravenously injected ^{131}I-labelled antigens and 3:5-diiodotyrosine in immune and normal rabbits. By A. E. Almond, G. E. Francis, J. D. Hawkins and A. Wormall xxxiii

Phosphorus metabolism in the housefly *Musca domestica*. By F. P. W. Winteringham, P. M. Loveday and G. C. Hellyer xxxiii

The stabilization of elastin by a polysaccharide. By G. C. Wood xxxiv

Studies on the complex nature of the elastin-elastase system. By D. A. Hall xxxv

Enzymic estimation of citric acid. By S. Dagley and E. A. Dawes xxxv

Production of keto acids from acetate by a vibrio. By S. Dagley and M. D. Patel xxxvi

The reactions of some haemoglobin derivatives with ^{131}I-labelled *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene. By H. Jackson and R. Thompson xxxvi

INDEX OF AUTHORS

	PAGE
Ahmad, K.	817
Aldridge, W. N.	763
Almond, A. E.	xxxiii
Ansell, G. B.	768
Baker, J. W.	xxxii
Barlow, A. J. E.	xxxi
Barry, J. M.	783
Bauld, W. S.	872
Bergmann, F.	888
Bieri, J. G.	xxxi
Boursnell, J. C.	745
Bowen, T. J.	766
Bowes, J. H.	735
Boyd, G. S.	892
Canivet, J.	867
Chattaway, F. W.	xxxi
Chen, Y.-T.	821
Clark, N. G.	839
Cohen, S. S.	774
Coombs, R. R. A.	745
Dagley, S.	xxxv, xxxvi
Davies, R. E.	880
Davison, A. N.	763
Dawes, E. A.	xxxv
Fernell, W. R.	758
Folkes, J. P.	721, 730
Francis, G. E.	xxxiii
Gale, E. F.	721, 730
Gooder, H.	xxxii
Goodwin, T. W.	834
Gordon, J. J.	812
Gregory, M. E.	830
Hall, D. A.	xxxv
Hams, A. F.	839
Hanes, C. S.	824
Happold, F. C.	xxxii
Hawkins, J. D.	xxxiii
Hele, M. P.	857, 864
Hellyer, G. C.	xxxiii
Holdsworth, E. S.	830
Hughes, D. E.	851
Isherwood, F. A.	821, 824
Jackson, H.	xxxvi
Karim, M. A.	817
Kerr, L. M. H.	872
King, H. K.	758
Klyne, W.	xxviii
Kogut, M.	800
Lewis, S. E.	xxvii
Loveday, P. M.	xxxiii
Mapson, L. W.	821
Morton, R. K.	786, 795
Moss, J. A.	735
Norman, J. M.	768
Norymberski, J. K.	876
Patel, M. D.	xxxvi
Perkins, D. J.	xxviii
Podoski, E. P.	800
Pollard, C. J.	xxxi
Reiss, O. K.	783
Rimington, C.	867
Rizk, V.	745
Robinson, E.	xxx
Sermin, A. T.	876
Shimoni, E.	888
Slater, E. C.	xxvii
Smith, M. J. H.	xxx
Stokes, W. M.	xxviii
Taylor, K. W.	xxx
Terner, C.	xxix
Thompson, C. C.	xxxi
Thompson, R.	xxxvi
Weil-Malherbe, H.	741
Whittam, R.	880
Williamson, D. H.	851
Winteringham, F. P. W.	xxxiii
Wood, G. C.	xxxiv
Wormall, A.	xxxiii
Wurzel, M.	888
Wyatt, G. R.	774

The Assimilation of Amino Acids by Bacteria

18. THE INCORPORATION OF GLUTAMIC ACID INTO THE PROTEIN FRACTION OF *STAPHYLOCOCCUS AUREUS*

By E. F. GALE and JOAN P. FOLKES
Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge

(*Received* 4 *May* 1953)

Melchior, Mellody & Klotz (1948) and Melchior, Klioze & Klotz (1951) have shown that incubation of washed cells of *Escherichia coli* with ^{35}S-labelled methionine results in an incorporation of the isotope into the cell protein. This incorporation was abolished by heating the cells to 100°, was stimulated by the addition of glucose and ammonium ions, and was inhibited by sodium azide and 2:4-dinitrophenol. A number of workers have investigated the incorporation of labelled amino acids into the proteins of tissue slices, 'homogenates', bone marrow or reticulocytes (Melchior & Tarver, 1947; Zamecnik & Frantz, 1950; Winnick (1950); Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1950, 1952; Kit & Greenberg, 1952; Siekevitz, 1952), and the question has arisen whether such incorporation involves protein synthesis, although net synthesis of protein has not been demonstrated and is improbable under many of the conditions used. The incorporation might be due to adsorption of labelled residues on to protein, but prolonged washing or dialysis has failed to remove the activity, and Borsook *et al.* (1952) have shown that labelled amino acids incorporated into the proteins of bone marrow or reticulocytes can be found in a variety of peptides obtained by partial hydrolysis of the proteins. Again, if carboxyl-labelled amino acids are used, treatment of the protein, after incorporation, with ninhydrin fails to remove the isotope, suggesting that the carboxyl group of the incorporated amino acid is no longer in the free state (Borsook *et al.* 1952). Holloway & Ripley (1952) have shown that the nucleic acid content of reticulocytes can be altered by injection of phenylhydrazine into rabbits some time before bleeding and that the incorporation of radioactive leucine into the protein of such reticulocytes can be correlated with their ribonucleic acid content. Doubt has been thrown on the biological significance of such studies by Brunish & Luck (1952), who have obtained incorporation of labelled amino acids into preparations of deoxyribonucleohistone and histone, and find that the rate of incorporation is increased by raising the temperature to 100°.

If washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) are incubated with glucose and glutamic acid, free glutamic acid becomes concentrated within the cells, but there is no increase of cellular protein (Gale, 1947*a*, *b*). If the cells are incubated with glucose and a mixture of amino acids, which must include all those essential for growth, then the accumulation of free glutamic acid within the cells ceases, protein synthesis takes place and glutamic acid from inside and outside the cell is incorporated quantitatively into the cell protein (Gale, 1951; Gale & Folkes, 1953*a*). The rate of protein synthesis can be correlated with the nucleic acid content of the cells (Gale & Folkes, 1953*a*), and is increased by the addition of purines and pyrimidines to the incubation mixture. Chloramphenicol, aureomycin and terramycin inhibit protein synthesis at bactericidal concentrations; bacitracin inhibits both protein and nucleic acid synthesis but at concentrations approximately 100 times that required to prevent growth, and penicillin at high concentrations prevents the stimulation of protein synthesis which is normally produced by the addition of purines and pyrimidines, while having little or no effect on the rate of protein synthesis occurring in the absence of such addition (Gale & Folkes, 1953*b*). It seemed that these experimental systems provided a means for the investigation of incorporation of labelled amino acids, and its relation to protein synthesis in *Staph. aureus* and the present paper presents results obtained with [^{14}C]glutamic acid.

METHODS

Organism. The organism used throughout this work was *Staphylococcus aureus* strain Duncan, which has been used for previous studies in this series (Gale & Folkes, 1953*a*, *b*).

Growth and preparation of suspensions. The organism was grown for 15 hr. at 30° in the 'deficient' medium consisting of a salt mixture, glucose, marmite and arginine (Gale, 1951), dispensed in Roux bottles. The cells were harvested on the centrifuge, washed once with water and made into washed suspension in distilled water; suspension density = 25·0 mg. dry weight of cells/ml. Suspension densities were deter-

mined on a Hilger absorptiometer previously calibrated against the organism used.

Incubation mixtures. Solutions of amino acids and the mixture A thereof were prepared as previously described (Gale & Folkes, 1953*a*). The radioactive glutamic acid used for the majority of the experiments was synthetic DL-[α-carboxy-^{14}C]glutamic acid of activity 0·9 mc/m-mole and was obtained from the Radiochemical Centre, Amersham. Some experiments, indicated in the text, were carried out with randomly labelled glutamic acid and other amino acids obtained biosynthetically. In general the radioactive amino acids were dissolved in buffered salt solution (Gale, 1947*a*) to a known strength or activity. Incubation mixtures were put up in 15 ml. centrifuge tubes; the following amounts of the various components were used and the final volume adjusted with water to 3·0 ml. in all cases: 0·2 ml. 20% (w/v) glucose, 0·3 ml. amino acid mixture A, 0·2 ml. washed suspension of *Staph. aureus* as above, 2·0 ml. buffered saline solution containing a known amount of radioactive amino acid, 0·2 ml. inhibitor solution; where any components were omitted an equivalent volume of water was substituted. The tubes were closed with rubber bungs and incubated in a water bath at 37°.

Preparation of protein fraction of cells for ^{14}C estimation. At the end of the incubation period, the reaction was stopped by the addition of 1·0 ml. 0·2M acetate buffer pH 4·5 to each tube, simultaneously immersing the tube in an ice bath. The tubes were then centrifuged at 1000 *g* for 10 min. and supernatant solutions withdrawn with a hypodermic syringe. The organisms were resuspended in approximately 5 ml. distilled water and again centrifuged; this washing procedure was repeated three times in all. After the third washing, the organisms were suspended in 2·0 ml. 5% (w/v) trichloroacetic acid (TCA) and placed in the ice chest for 2 hr. The precipitates were then centrifuged down at 1000 *g* for 5 min., washed once in 5% TCA, once in 5% acetic acid and once in 1% acetic acid. Each precipitate was suspended in 0·75 ml. 20% (v/v) aqueous acetone and 0·3 ml. samples, in duplicate, placed on polythene disks of surface area 2 sq.cm. One drop of 1% (w/v) polyvinyl alcohol was added to each sample and the preparations dried *in vacuo*. In this way a film of protein corresponding to 1·0 mg. dry weight of organism/sq.cm. was obtained on each disk.

Precipitates prepared in this way contain nucleic acid as well as protein. When any doubt existed concerning possible incorporation of isotope into nucleic acid as well as protein, the nucleic acid was removed from the sample by three consecutive extractions with 5% TCA at 90° for 10 min. (Gale & Folkes, 1953*a*) carried out after the first precipitation with TCA. When preparations were treated in this way, results are expressed in terms of the 'protein fraction'; when the extraction was omitted, results are expressed in terms of 'TCA precipitate'. 1·0 mg. dry weight of cells yields 0·65 mg. TCA precipitate or 0·49 mg. 'protein fraction'.

The ^{14}C content of the dried preparations was estimated by use of an end-window Geiger-Müller tube and scaler in the conventional manner. The apparatus was calibrated against known amounts of DL-[α-carboxy-^{14}C]glutamic acid; 1·0 mμmole gave 44 counts/min., and where the radioactivity of protein fractions was due solely to glutamic acid, activities have been expressed in terms of mμmoles glutamic acid incorporated.

Estimation of radioactivity of free glutamic acid within the cells. Treatment of cell suspensions with TCA (as above) results in liberation of the free glutamic acid from the cells into the supernatant liquid. Attempts were made to collect the supernatant and estimate the ^{14}C content after taking to dryness *in vacuo*. It was found that such films, even if treated with ether to remove the bulk of the TCA, tended to absorb water during the counting procedure with the result that counts were variable and subject to error. Salton (1951) showed that the free glutamic acid of *Staph. aureus* is released if the cells are treated with cetyltrimethylammonium bromide (CTAB). Accordingly, cells were suspended in water containing CTAB (100 μg./mg. dry weight of cells), incubated for 30 min. at 37° and the cells then removed on the centrifuge. The cells were washed in water (0·1 ml./5 mg. dry weight of cells) and the washings added to the CTAB extract. The CTAB extract was then evaporated to dryness, taken up in approx. 0·3 ml. water, transferred to a polythene disk and prepared for counting as above.

The total free glutamic acid content of the cells was estimated manometrically by use of the glutamic acid decarboxylase (Gale, 1945, 1947*a*).

Determination of the distribution of ^{14}C amongst the amino acids of the cell protein. Samples of the protein fraction corresponding to 6 mg. dry weight of cells were hydrolysed for 20 hr. in 0·4 ml. 6N-HCl in sealed tubes at 105°, evaporated to dryness *in vacuo*, taken up in approx. 0·5 ml. water and evaporated to dryness three successive times, taken up in 30 μl. water and 10 μl. used for spotting a paper chromatogram. Chromatograms were run in two dimensions using as solvents phenol:ammonia (80% phenol, 20% water and 0·3% ammonia solution (sp.gr. 0·880)) and *sec.*-butanol: formic acid:water in the proportions 70:10:20, v/v. After drying, the papers were placed in contact with X-ray-sensitive film (Ilford Ilfex) and the films developed, one after 3 days' contact, and a second after 14 days' contact to bring out any weakly radioactive spots. Active spots were located from the radioautographs, cut out of the paper, eluted and the eluate evaporated before counting as usual.

RESULTS

Incubation of washed suspensions of *Staph. aureus* with glucose and ^{14}C-labelled glutamic acid resulted in the appearance of radioactivity in the protein fraction of the cells whether other amino acids were present in the incubation mixture or not. The activity associated with the protein fraction was not significantly decreased by washing the protein precipitate three to five times with water, 5% TCA or 1% acetic acid, nor was there any loss of activity as a result of treatment of the precipitate at pH 2·7 in citrate buffer saturated with ninhydrin. There was no incorporation of activity if the cells were heated to 100° for 10 min. before incubation, and the incorporation was reduced to insignificant values if the incubation mixture contained 0·01M 2:4-dinitrophenol. Omission of glucose from the incubation mixture reduced the incorporation to less than 10% of that occurring in its presence. It appeared that an incorporation of ^{14}C into the protein fraction was occurring in these experiments and that the general properties of the process were similar to those previously reported by other workers for other cells (Melchior *et al.* 1948; Melchior *et al.* 1951).

Effect of amino acid mixture A on the rate of incorporation. Fig. 1 shows the rate of incorporation of ^{14}C into the TCA-precipitable fraction of washed *Staph. aureus* when the cells are incubated with glucose and (1) [^{14}C]glutamic acid or (2) a complete mixture of amino acids and [^{14}C]glutamic acid. In condition 2, net synthesis of cell protein occurs (Gale & Folkes, 1953*a*), and it can be seen that the rate and amount of ^{14}C incorporation are markedly greater than in condition 1 where net synthesis has not been demonstrated. If nucleic acid is not extracted from the TCA precipitates, the radioactivity is 8–11% higher under condition 1 and unchanged within experimental error under condition 2; this agrees with previous work which indicated that, when protein synthesis is taking place, glutamic acid is quantitatively taken up into the protein (Gale, 1951; Gale & Folkes, 1953*a*).

Distribution of ^{14}C amongst the amino acids of the cell protein. Samples of protein from cells which have been incubated with [α-carboxy-^{14}C]glutamic acid under conditions 1 and 2 of Fig. 1 have been hydrolysed, run on paper chromatograms and radioautographed as described above. In one experiment the amount of radioactivity placed on the paper corresponded to 652 counts/min. from cells incubated under condition 1, and 5530 counts/min. from cells incubated under condition 2. After exposure of the chromatogram to X-ray-sensitive film for 3 days, the radioautographs showed, for both conditions, one spot only and that in a position corresponding to glutamic acid. After further exposure for 14 days, no further spots were visible on the paper corresponding to condition 1 but a faint spot in a position corresponding to leucine/isoleucine appeared on the paper for condition 2. After elution of the paper corresponding to the active glutamic acid spot, 83% of the activity initially added was recovered for condition 1, and 86% for condition 2, while recovery of control samples of ^{14}[C]glutamic acid was 87 and 88%. Elution of the paper corresponding to the faintly active 'leucine' spot in condition 2 gave a further recovery of activity equal to 1·1% of the initial activity.

Effect of concentration of organism. Experiments were carried out in which the suspension density of the cells in the incubation mixture varied from 0·8 to 6·7 mg. dry weight/ml. Fig. 2 shows that the incorporation/mg. TCA precipitate falls if the suspension density exceeds 5·0 mg. dry weight/ml. under condition 1 or 2·0 mg. dry weight/ml. under condition 2. In all other experiments, the suspension density used was 1·7 mg. dry weight of cells/ml.

Effect of glutamic acid concentration. The rate of incorporation varies with the concentration of glutamic acid present for low values of the latter, but becomes independent of concentration when this is greater than 0·9 μmole/ml. under condition 1 or 3·3 μmoles/ml. under condition 2. The curves relating rate of incorporation to glutamic acid concentration resemble those obtained for the variation of enzyme velocity with substrate concentration

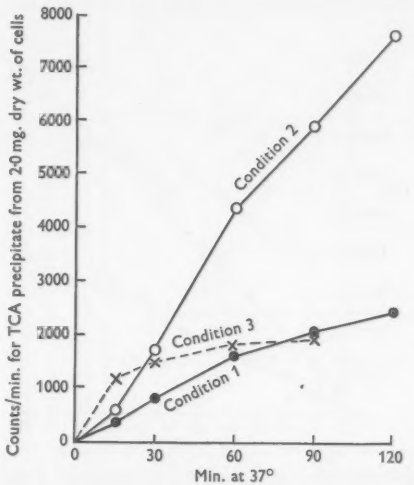


Fig. 1. Incorporation of radioactivity into the TCA-precipitable fraction of *Staphylococcus aureus* during incubation with ^{14}C-labelled glutamic acid. Washed suspension of *Staph. aureus* incubated at 37° and final suspension density = 1·7 mg. dry weight of cells/ml. in buffered saline solution containing 0·75% (w/v) glucose and 1·0 μmole [^{14}C]glutamic acid (specific activity 0·9 mc/m-mole)/ml. with the following additions. Condition 1, no addition; condition 2, addition of amino acid mixture A, final concentration of each component = 0·2 mg./ml.; condition 3, as condition 2 with omission of aspartic acid.

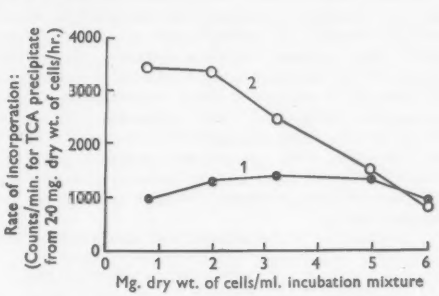


Fig. 2. Variation of incorporation of radioactivity with suspension density of *Staph. aureus* during incubation. Conditions as for Fig. 1 but final suspension density of washed cells varied as shown above; samples removed after 1 hr. at 37°.

and can be treated by the method of Lineweaver & Burk (1934). Fig. 3 shows the reciprocal of the rate of incorporation plotted against the reciprocal of glutamic acid concentration; calculation of the 'apparent Michaelis constant' for the incorporation process shows that the value of 0·21 μmole glutamic acid/ml. is obtained under both conditions 1 and 2.

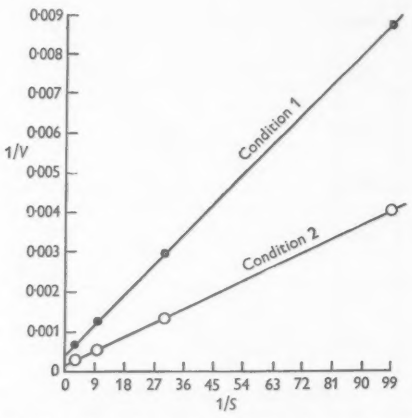


Fig. 3. Variation of velocity of incorporation of radioactivity with concentration of [^{14}C]glutamic acid; velocity and concentration plotted as reciprocals. Conditions as for Fig. 1, samples removed after 1 hr. at 37°. S = μmole [^{14}C]glutamic acid (specific activity 0·9 mc/m-mole)/ml. V = counts/min. of TCA precipitate corresponding to 2·0 mg. dry weight of cells, after 1 hr. incubation.

Table 1. *Effect of temperature on the incorporation of glutamic acid into the TCA precipitate from* Staphylococcus aureus

(Washed suspensions of *Staph. aureus* Duncan (1·7 mg. dry wt. of cells/ml.) incubated at temperatures shown below for 60 min. in buffered saline solution containing 0·75% (w/v) glucose, 1·0 μmole glutamic acid (specific activity 0·2 mc/m-mole)/ml. with and without the addition of amino acid mixture A (final concentration of each component = 0·2 mg./ml.). After incubation, cells washed three times in water, precipitated in the cold with 5% trichloroacetic acid; precipitates washed, dried and counted. Results expressed as mμmole glutamic acid incorporated/TCA precipitate obtained from 2·0 mg. dry weight of cells.)

Temp. (°)	Glutamic acid incorporated: Amino acid mixture A absent	Glutamic acid incorporated: Amino acid mixture A present
25	9·5	11·1
35	21·7	47·5
44	32·0	89·0
60	0·8	0·6
100	0·4	0·2

To economize in the use of isotope, experiments have usually been carried out with 0·2 μmole [^{14}C]glutamic acid/ml. diluted with 0·8 μmole [^{12}C]glutamic acid/ml. to saturate the incorporation mechanism.

Effect of temperature. Table 1 shows the rate of incorporation obtained under condition 1 or 2 over a range of temperatures. The rate increases as the temperature rises to 40–44° but incorporation ceases at temperatures above 60°. The process is consequently different from that studied by Brunish & Luck (1952) in nucleohistone preparations where incorporation was greater at 100° than at 37°. It can be seen from Table 1 that the temperature coefficient for incorporation under condition 2 is greater than that under condition 1.

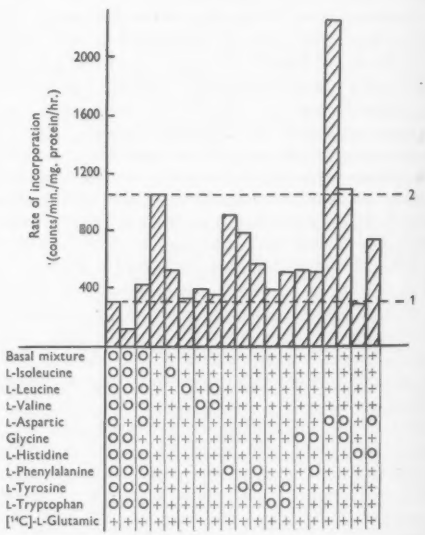


Fig. 4. Variation of rate of incorporation of radioactivity into the protein fraction of *Staphylococcus aureus* incubated with [^{14}C]glutamic acid and mixtures of unlabelled amino acids. Washed suspension of *Staph. aureus* incubated for 1 hr. at 37° and final suspension density = 1·7 mg. dry wt. of cells/ml., in buffered saline containing 0·75% (w/v) glucose, 1·0 μmole [^{14}C]glutamic acid (specific activity 0·2 mc/m-mole)/ml. and amino acid mixtures as shown (+ = amino acid present; ○ = amino acid absent), each component at final concentration = 0·2 mg./ml. Basal mixture contains lysine, arginine, alanine, proline, cysteine, methionine, threonine, serine, at final concentration of L-isomer in each case = 0·2 mg./ml. Samples removed after 1 hr. incubation and protein fractions prepared for counting. 1 = Rate of incorporation when glutamic acid is the only amino acid present (condition 1); 2 = rate of incorporation when a complete mixture of amino-acids is present (condition 2).

Effect of other amino acids on glutamic acid incorporation. The incorporation studies so far described were carried out either with glutamic acid as the only amino acid present (condition 1) or with a complete mixture of amino acids present (condition 2). The effect was tried of adding amino acids singly and in mixtures to the incubation mixture containing glutamic acid. Most of the naturally occurring amino acids have little or no effect on glutamic acid incorporation when added singly although, as shown in Fig. 4, aspartic acid decreases while glycine increases the incorporation. The effect was next tested of adding the complete mixture A but omitting one essential amino acid; Fig. 4 shows that omission of phenylalanine or tyrosine has little effect, whereas omission of leucine, valine, tryptophan or histidine reduces the incorporation to that level obtained when glutamic acid is the only amino acid present. The effect of omitting aspartic acid is surprising in that the initial rate of glutamic acid incorporation is 230% of that obtained in the presence of the complete mixture, while the effects of omission of either glycine or histidine are largely overcome by the further omission of aspartic acid.

Fig. 1 shows that the effect of amino acid mixture A without aspartic acid is to increase the rate of glutamic acid incorporation during the initial stages of incubation only, and that the rate then decreases so that the amount of incorporation after 90 min. incubation is no greater than that obtained when glutamic acid is the only amino acid added. When the complete amino acid mixture A is present, the initial rate of incorporation is between that obtained for glutamic acid alone and that obtained for the mixture without aspartic acid, but this rate is maintained throughout the incubation period.

Actions of antibiotics on glutamic acid incorporation

Chloramphenicol. Fig. 5 shows the action of chloramphenicol on glutamic acid incorporation when incubation takes place in the absence (condition 1) and presence (condition 2) of amino acid mixture A. Under condition 2 the incorporation is highly sensitive to chloramphenicol, 85% inhibition occurring in the presence of that concentration of the antibiotic which prevents growth of the organism. The incorporation that takes place under condition 1 is much less sensitive, 30 μg. chloramphenicol/ml. producing approximately 15% inhibition only. It can be seen from Fig. 5 that the rate of incorporation under condition 2 in the presence of high concentrations of chloramphenicol is less than that which occurs under condition 1 in the same antibiotic concentrations.

Aureomycin. Fig. 6 shows that aureomycin at low concentrations differentiates between glutamic

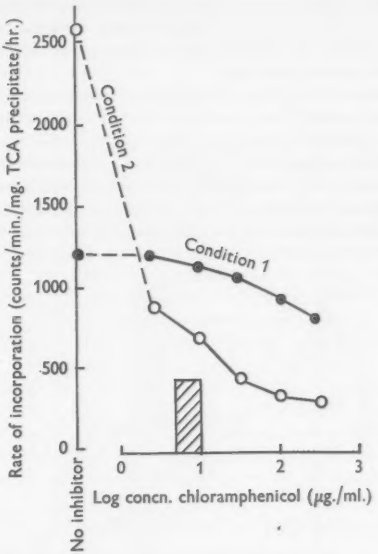


Fig. 5. Effect of chloramphenicol on rate of incorporation. Conditions as for Fig. 1 with addition of chloramphenicol as indicated; samples taken after 1 hr. at 37°. Cross-hatching indicates range of drug preventing growth in a complete medium inoculated with approx. 10^6 cells/5·0 ml.

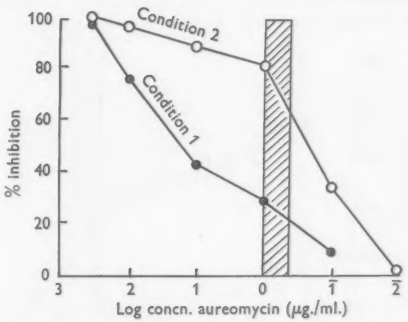


Fig. 6. Effect of aureomycin on the incorporation of [^{14}C]glutamic acid in washed suspensions of *Staphylococcus aureus*. Conditions as for Fig. 1, with aureomycin added as indicated; inhibitions calculated as percentage of rate obtained in absence of aureomycin (=952 counts/min/mg. protein fraction in condition 1, or 3034 counts/min./mg. protein fraction in condition 2). Cross-hatching indicates range of drug preventing growth in a complete medium inoculated with approx. 10^6 cells/5·0 ml.

acid incorporation under condition 1 and condition 2. At concentrations higher than 300 μg. aureomycin/ml. incorporation is completely inhibited under both conditions.

Bacitracin. When glutamic acid is the only amino acid present, bacitracin at the growth-inhibitory concentration produces 50 % inhibition of the rate of incorporation. The shape of the inhibition curve, shown in Fig. 7, suggests that the process is a complex one involving at least two parts, one of which is completely inhibited at the bactericidal concentration of the antibiotic. At low concentrations, bacitracin has little effect on incorporation under condition 2 but the process, whether under condition 1 or 2, is completely inhibited at antibiotic concentrations approximately 100 times the bactericidal level.

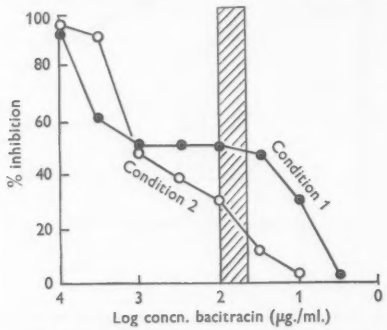


Fig. 7. Effect of bacitracin on the incorporation of [^{14}C]-glutamic acid by washed suspensions of *Staphylococcus aureus*. Conditions as for Fig. 1 with bacitracin added as indicated and concentration of [^{14}C]glutamic acid = 1·0 μmole (specific activity 0·2 mc/m-mole)/ml. Inhibition calculated as percentage rate in absence of bacitracin (= 483 in condition 1 and 1099 in condition 2, counts/min./mg. protein fraction). Cross-hatching indicates range of bacitracin concentration preventing growth in a complete medium inoculated with approx. 10^6 cells/5·0 ml.

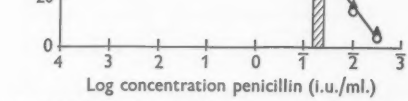


Fig. 8. Inhibition of [^{14}C]glutamic acid incorporation into the protein fraction of *Staphylococcus aureus* by penicillin. Details as for Fig. 1, condition 1. Samples taken after 60 min. incubation and inhibition determined as percentage of that incorporation obtained in the absence of penicillin. Points of different shape indicate different experiments with concentrations of [^{14}C]glutamic acid = 0·1 (×, ▲) or 1·0 (○, ●) μmole/ml.

Penicillin. Penicillin has no effect on glutamic acid incorporation under condition 2 until the antibiotic concentration is raised to approximately 10 000 times the growth inhibitory level. Fig. 8 shows, however, that bactericidal concentrations produce 45–50 % inhibition of the incorporation that occurs under condition 1, and that the curve shows an increase of only 10 % in the inhibition for an increase of penicillin from 0·1 to 100 international units/ml.

The actions of penicillin and bacitracin appear to be somewhat similar in that both give rise to approximately 50 % inhibition of glutamic acid incorporation under condition 1 when tested at the growth-inhibitory level. Table 2 shows that the two antibiotics have a synergic effect when added together at subbactericidal concentrations but not at concentrations corresponding to the plateaux in their inhibition curves.

Table 2. *Actions of penicillin and bacitracin on the incorporation of glutamic acid into the protein fraction of* Staphylococcus aureus

(Conditions as for Table 1 with penicillin or bacitracin as below; temp. 37°. Protein fraction prepared after incubation, dried and counted.)

Penicillin (i.u./ml.)	Bacitracin (μg./ml.)	Glutamic acid incorporated (mμmole/mg. protein)	Inhibition of glutamic acid incorporation (%)
0·0	0·0	26·1	—
0·03	0·0	21·4	18
0·0	3·0	26·4	0
0·03	3·0	17·7	32
1·0	0·0	15·8	39
0·0	30·0	12·8	51
1·0	30·0	10·8	58
10·0	0·0	14·9	43
0·0	100·0	12·0	54
10·0	100·0	11·15	57

Non-competitive nature of the inhibitions. In the following paper (Gale & Folkes, 1953*c*) it is shown that the incorporation of phenylalanine into the protein of *Staph. aureus* is competitively inhibited by *p*-chlorophenylalanine, and non-competitively inhibited by aureomycin and chloramphenicol. Similar experiments have been carried out with the antibiotics and glutamic acid incorporation under the conditions described above; in all cases the inhibition proved to be non-competitive.

Radioactivity of free glutamic acid within Staph. aureus *during incorporation into protein*

Washed suspensions of *Staph. aureus* were incubated with [^{14}C]glutamic acid and glucose, samples withdrawn during a 90 min. incubation at 37°, and

the following estimations carried out: (1) free glutamic acid content of the cells, (2) radioactivity of free glutamic acid within the cells, and (3) radioactivity of the protein fraction obtained from the cells. The relative specific activity of the free glutamic acid was calculated from (1) and (2). The experiment was repeated with the addition to the incubation mixture of either 1·0 mg. bacitracin/ml. or 0·3 international unit penicillin/ml. The presence of either penicillin or bacitracin has no effect on the accumulation of free glutamic acid within the cells, as previously shown (Gale & Taylor, 1947; Paine, 1951), but Fig. 9 shows that the antibiotics decrease the rate of glutamic acid incorporation into the protein fraction of the cells. In the absence of antibiotics, the specific activity of the free glutamic acid within the cells rises during the early stages of the incubation as the radioactive glutamic acid is taken into the cells; it then decreases as the activity of the protein increases. In the presence of either bacitracin or penicillin, the specific activity of the free glutamic acid rises to a slightly higher value than in the absence of antibiotics, and the decrease in specific activity during the later stages of incubation is reduced or, in the case of 1·0 mg. bacitracin/ml., abolished. The concentration of total free glutamic acid within the cells remains constant after the first 15 min. and is unaffected by the presence of either antibiotic.

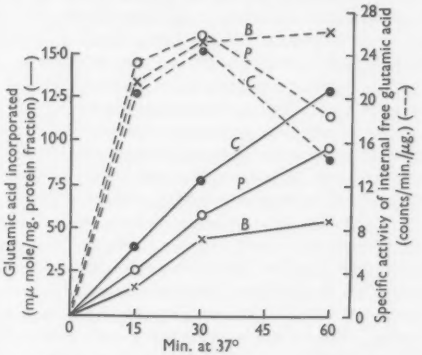


Fig. 9. Effect of penicillin and bacitracin on the incorporation of [^{14}C]glutamic acid into the protein fraction of *Staphylococcus aureus* (continuous lines) and on the specific activity of the free glutamic acid accumulated within the cells (broken lines). Washed suspension of *Staph. aureus* Duncan incubated in buffered saline solution containing 0·75% glucose, 0·1 μmole [^{14}C]-glutamic acid (specific activity 0·9 mc/m-mole)/ml. and (*C*) no addition, (*P*) 0·3 i.u. of penicillin/ml., (*B*) 1·0 mg. bacitracin/ml. Samples taken after times shown. Incorporation measured on protein fractions of the cells; intact cells assayed for free glutamic acid by the decarboxylase method; free glutamic acid released from cells by treatment with CTAB, dried and counted. Specific activity of free glutamic acid calculated as counts/min./μg. free glutamic acid.

Actions of antibiotics on the incorporation of other amino acids

It is important to know to what extent the results described above for the incorporation of glutamic acid can be extended to other amino acids. Small amounts of amino acids randomly labelled with ^{14}C were obtained by biosynthesis and the ability of washed suspensions of *Staph. aureus* to incorporate these amino acids was tested. Insufficient material

Table 3. *Action of antibiotics on the incorporation of amino acids into the protein fraction of* Staphylococcus aureus *Duncan*

(Washed suspensions of *Staph. aureus* (final suspension density = 1·7 mg. dry wt. of cells/ml.) incubated for 60 min. at 37° in buffered saline containing 0·75% (w/v) glucose and a radioactive amino acid at the concentration shown below, in the presence and absence of antibiotics as shown below. Amino acids other than cystine were randomly labelled with ^{14}C; cystine used was ^{35}S-labelled. Protein fractions prepared after incubation, dried and counted.)

Amino acid	Radioactivity of incubation medium (counts/min./ml.)	Radioactivity of protein fraction (counts/min./mg.)	Inhibition of incorporation (%)				
			Penicillin (i.u./ml.)			Bacitracin (100 μg./ml.)	Chloramphenicol (100 μg./ml.)
			0·03	0·3	30·0		
Glutamic acid	2750	477	25	47	53	63	20
Glycine	5000	1213	8	22	37	26	12
Alanine	1960	175	6	12	15	20	21
Aspartic acid	2140	199	2	10	11	18	33
Isoleucine/leucine	5333	1277	—	4	15	17	60
Threonine	1400	127	—	2	15	15	33
Phenylalanine	4869	502	—	5	11	0	62
Lysine	1773	547	—	2	4	0	33
Arginine	1925	161	—	2	3	0	54
Methionine/valine	2400	300	—	0	1	0	57
Tyrosine	603	281	—	0	0	0	55
Proline	973	28	0	0	0	0	70
[^{35}S]Cystine	1375	26	0	10	25	22	0

was available for the complete tests described for glutamic acid to be carried out but, in all cases tested, it was found that incubation of the cells with an amino acid and glucose resulted in radioactivity appearing in the protein fraction of the cells. The effect of penicillin, bacitracin and chloramphenicol was tested on this incorporation in each case, and the results are summarized in Table 3. Penicillin was tested at three concentrations, one below and two on the plateau obtained in the curve for inhibition of glutamic acid incorporation. Table 3 shows that there are marked differences in the sensitivity of the incorporation processes for various amino acids; some such as tyrosine, proline, lysine, arginine and the methionine/valine mixture being unaffected by penicillin. Glutamic acid is the most sensitive, followed by glycine and alanine, but the glycine incorporation is only approximately half as sensitive as glutamic acid incorporation. The activities of penicillin and bacitracin, at 'plateau concentrations', are very similar for all amino acids tested. The sensitivity of the processes towards chloramphenicol varies again, and there is a suggestion that those processes which are least sensitive to penicillin are most sensitive to chloramphenicol, and vice versa.

DISCUSSION

The results presented here demonstrate clearly that the processes whereby glutamic acid is incorporated into the protein fraction of *Staph. aureus* differ according to the conditions of incubation. It has been shown previously that washed suspensions of *Staph. aureus* will synthesize protein if they are incubated with glucose and a mixture of the amino acids essential for growth (Gale & Folkes, 1953*a*). Such protein synthesis is almost completely inhibited by concentrations of chloramphenicol or aureomycin which prevent growth (Gale & Folkes, 1953*b*). Under similar conditions (condition 2) radioactive glutamic acid is rapidly incorporated into the protein fraction of the cells and this incorporation is inhibited 85–90 % by chloramphenicol or aureomycin at the growth-inhibitory concentrations. So far it would appear that the incorporation of [^{14}C]glutamic acid can be taken as an indication of protein synthesis. However, if we alter the amino acid composition of the incubation mixture, we still obtain incorporation although net protein synthesis may no longer occur, and there would appear to be no correlation between the rate of glutamic acid incorporation, particularly during the early stages of incubation, and the rate of increase in cell protein.

There are very marked differences in the sensitivities of incorporation processes to antibiotics depending upon whether conditions are suitable for protein synthesis or not. Thus incorporation under condition 1 is less sensitive to chloramphenicol and aureomycin, and more sensitive to bacitracin and penicillin, than incorporation under condition 2. Under condition 2 glutamic acid is taken up from the medium and added into the new protein directly and quantitatively (Gale, 1951; Gale & Folkes, 1953*a*); under condition 1 there is no increase in cell protein, but free glutamic acid accumulates within the cells (Gale, 1947*a*). Investigation of the specific activity of the free glutamic acid within the cells shows that, once the initial period of uptake is over, an increase in activity of the cell protein is accompanied by a decrease in activity of the free glutamic acid within the cell. Inhibition of the incorporation into the protein prevents the decrease in specific activity of the free glutamic acid. Since the concentration of total free glutamic acid within the cells remains constant during these changes, it would appear that the incorporation of radioactive glutamic acid into the cell protein occurs as a result of an exchange between the free glutamic acid molecules in the cell and glutamic acid residues in the protein. To prove that such an exchange does occur it would be necessary to show that active residues in the protein could be made to exchange with unlabelled free glutamic acid. Attempts to do this with intact cells have not been successful since the highly efficient concentration of free glutamic acid within the cells during the incorporation process has made it technically almost impossible to reverse the situation. However, such a reversal has been accomplished with cells damaged by supersonic vibration (Gale, 1953) and this will be reported later.

It would appear that glutamic acid incorporation can occur as a result of exchange reactions with cell proteins, and that the rate of such reactions can be greatly influenced by the presence of other amino acids. When, however, the medium contains all the amino acids necessary for protein synthesis to occur, there is a change in the reactions within the cell which results in glutamic acid (and other amino acids) being formed into new protein. Since high concentrations of chloramphenicol suppress incorporation during protein synthesis to a level well below that occurring during simple exchange, and since penicillin inhibits exchange incorporation but has no effect on incorporation during protein synthesis, it would seem that exchange reactions must be suppressed when protein synthesis begins. Whatever may be the final interpretation of these findings, it is clear that it is dangerous to use incorporation studies as measures of protein synthesis in living cells.

There have been many effects ascribed to penicillin in the past, but some of these can be demonstrated only if the cells are allowed to grow in the

presence of the antibiotic for a short time, e.g. inhibition of glutamic acid uptake (Gale & Taylor, 1947), or of guanosine oxidation (Gros, Beljanski & Macheboeuf, 1951), while others require very high concentrations of penicillin to show significant inhibitions, e.g. inhibition of ribonucleic acid breakdown (Krampitz & Werkman, 1947). The effect described here is the first that has been obtained with bactericidal concentrations of penicillin acting on washed suspensions of cells. At present it is difficult to interpret the effect. Penicillin appears to prevent exchange reactions which occur between cell proteins and certain amino acids in the amino acid pool of the cells. Of the amino acids affected, glutamic acid is the most sensitive; some amino acids such as proline, tyrosine and lysine are not affected at all. The shape of the curves obtained for the inhibition of glutamic acid incorporation by penicillin or bacitracin suggest that the reaction is a complex one; it may be that it is exchange with certain proteins only which is affected by penicillin. Such an action would presumably have no immediate effect on the enzymic activities of the non-growing cell but these proteins would be eliminated from the dynamic pool of the cell. Consequently when growth was resumed, enzymic activities due to these proteins would be lost, and this may be the explanation of the secondary effects mentioned above.

SUMMARY

1. When washed suspensions of *Staphylococcus aureus* are incubated with glucose and ^{14}C-labelled glutamic acid (condition 1), the protein fraction of the cells becomes progressively more radioactive. The radioactivity is associated with the glutamic acid residues of the protein.

2. The rate of this glutamic acid incorporation is increased by the addition of a mixture of amino acids containing all those essential for growth (condition 2). Omission of certain essential amino acids, such as histidine or tryptophan, results in the rate of incorporation falling to that obtained in condition 1. Omission of aspartic acid results in a greatly increased rate of incorporation which lasts for 30–60 min. and then falls to that under condition 1.

3. The incorporation is abolished if the reaction temperature rises to 60° or over.

4. Incorporation under condition 1 is markedly less sensitive to inhibition by chloramphenicol or aureomycin, and markedly more sensitive to inhibition by penicillin or bacitracin, than incorporation under condition 2.

5. The incorporation of glutamic acid into cell protein under condition 1 is accompanied by a decrease in the specific activity but not the total amount of the free glutamic acid within the cells. Inhibition of incorporation by penicillin or bacitracin does not affect the total amount of free glutamic acid within the cells, but prevents the decrease in its specific activity.

6. The processes involved in the incorporation of a variety of radioactive amino acids show differing sensitivities to penicillin, bacitracin and chloramphenicol. The process most sensitive to penicillin is that involved in the incorporation of glutamic acid.

The authors are indebted to Dr K. McQuillen for preparing the radioautographs, and to Mr M. K. Gaitonde for a gift of ^{35}S-labelled cystine.

REFERENCES

Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. (1950). *J. biol. Chem.* **186**, 297.
Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G. & Lowy, P. (1952). *J. biol. Chem.* **196**, 669.
Brunish, R. & Luck, J. M. (1952). *J. biol. Chem.* **197**, 869.
Gale, E. F. (1945). *Biochem. J.* **39**, 46.
Gale, E. F. (1947*a*). *J. gen. Microbiol.* **1**, 53.
Gale, E. F. (1947*b*). *J. gen. Microbiol.* **1**, 327.
Gale, E. F. (1951). *Biochem. J.* **48**, 290.
Gale, E. F. (1953). *6th Int. Congr. Microbiology, Symp. on Bacterial Metabolism.*
Gale, E. F. & Folkes, J. P. (1953*a*). *Biochem. J.* **53**, 483.
Gale, E. F. & Folkes, J. P. (1953*b*). *Biochem. J.* **53**, 493.
Gale, E. F. & Folkes, J. P. (1953*c*). *Biochem. J.* **55**, 730.
Gale, E. F. & Taylor, E. S. (1947). *J. gen. Microbiol.* **1**, 314.
Gros, F., Beljanski, M. & Macheboeuf, M. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1696.
Holloway, B. W. & Ripley, S. H. (1952). *J. biol. Chem.* **196**, 695.
Kit, S. & Greenberg, D. M. (1952). *J. biol. Chem.* **194**, 377.
Krampitz, L. O. & Werkman, C. H. (1947). *Arch. Biochem.* **12**, 57.
Lineweaver, H. & Burk, B. (1934). *J. Amer. chem. Soc.* **56**, 658.
Melchior, J. B., Klioze, O. & Klotz, I. M. (1951). *J. biol. Chem.* **189**, 411.
Melchior, J. B., Mellody, M. & Klotz, I. M. (1948). *J. biol. Chem.* **174**, 81.
Melchior, J. B. & Tarver, H. (1947). *Arch. Biochem.* **12**, 309.
Paine, T. F. jun. (1951). *J. Bact.* **61**, 259.
Salton, M. R. J. (1951). *J. gen. Microbiol.* **5**, 391.
Siekevitz, P. (1952). *J. biol. Chem.* **195**, 549.
Winnick, T. (1950). *Arch. Biochem.* **27**, 65.
Zamecnik, P. C. & Frantz, I. D. (1950). *Cold Spr. Harb. Symp. quant. Biol.* **14**, 199.

The Assimilation of Amino Acids by Bacteria

19. THE INHIBITION OF PHENYLALANINE INCORPORATION IN *STAPHYLOCOCCUS AUREUS* BY CHLORAMPHENICOL AND *p*-CHLOROPHENYLALANINE

By E. F. GALE and JOAN P. FOLKES
Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge

(*Received* 4 *May* 1953)

In the previous paper of this series (Gale & Folkes, 1953*c*) it was shown that incubation of washed suspensions of *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) with glucose and [^{14}C]-glutamic acid resulted in incorporation of the labelled glutamic acid into the protein fraction of the cells. This process occurred in the absence of demonstrable protein synthesis and underwent considerable modification in properties when a complete mixture of amino acids was added. Under these conditions net synthesis of protein took place and was inhibited by bactericidal concentrations of chloramphenicol (Gale & Folkes, 1953*a*, *b*). The incorporation of labelled glutamic acid was equally sensitive to the antibiotic as long as a complete mixture of amino acids was present but became markedly less sensitive when glutamic acid was the only amino acid present in the incubation mixture.

Woolley (1951) found that phenylalanine would decrease the antibacterial action of chloramphenicol when this was present in concentrations which partially inhibited growth, although the amino acid was ineffective as antagonist when growth inhibition was complete. He pointed out that there are structural similarities between chloramphenicol and phenylalanine, and suggested that the antibiotic acts as an inhibitor of phenylalanine metabolism and may prevent the incorporation of phenylalanine into proteins. Halvorson & Spiegelman (1952) investigated the inhibition of growth and enzyme formation in yeast by amino acid analogues, and found that certain derivatives of phenylalanine, *o*- and *p*-fluorophenylalanine in particular, were potent inhibitors whose action could be competitively antagonized by the corresponding amino acid only. It was thought that a study of the actions of chloramphenicol and an analogue of phenylalanine on the incorporation of that amino acid into the protein fraction of *Staph. aureus* might throw light on the relationship between amino acid incorporation and protein synthesis, and also on the mode of action of chloramphenicol.

METHODS

The organism, methods of growth, preparation of suspensions, incubation mixtures and conditions, preparation and counting of radioactive protein fractions were the same as those described in the previous paper (Gale & Folkes, 1953*c*). The radioactive phenylalanine, obtained from the Radiochemical Centre, Amersham, was DL-[β-^{14}C]phenylalanine of specific activity 1·19 mc/m-mole. The amino acid mixture used was that described as mixture A by Gale & Folkes (1953*a*) with the omission of glutamic acid and phenylalanine; these were added in labelled or non-labelled form as described below. Gale & Folkes (1953*c*) showed that the rate of incorporation of glutamic acid into the protein of *Staph. aureus* varied with the concentration of glutamic acid in a manner similar to that obtained for the variation of the rate of an enzyme-catalysed reaction with substrate concentration, and that a straight line was obtained if the reciprocal of the rate was plotted against the reciprocal of glutamic acid concentration. A similar situation has been found in the present studies and it has been possible to study the inhibition of incorporation by application of the methods of analysis devised by Lineweaver & Burk (1934).

RESULTS

Incorporation of phenylalanine into the protein fraction of Staph. aureus. Incubation of washed suspensions of *Staph. aureus* with glucose and ^{14}C-labelled phenylalanine (condition 1) results in a progressive incorporation of ^{14}C into the protein fraction of the cells in a manner similar to that previously described for glutamic acid (Gale & Folkes, 1953*c*). The addition of a complete mixture of amino acids (condition 2) markedly increases the rate of incorporation. Under both conditions the incorporation is prevented by prior heating of the cells to 100° for 10 min.

Effect of phenylalanine concentration on the rate of incorporation. Fig. 1 shows the effect of plotting the reciprocal of the rate of ^{14}C incorporation into the protein fraction against the reciprocal of the concentration of [^{14}C]phenylalanine present during incubation under conditions 1 and 2. The 'apparent Michaelis constant' for incorporation under con-

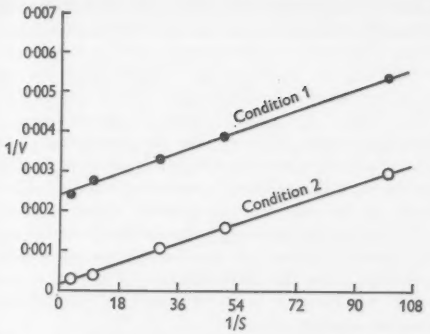


Fig. 1. Relation between rate of incorporation and phenylalanine concentration: incorporation of phenylalanine by *Staph. aureus*. S = μmole DL-[^{14}C]phenylalanine/ml. V = rate of incorporation into protein fraction expressed in counts/min./mg. protein fraction of cells after 1 hr. incubation at 37°. Washed suspension of *Staph. aureus* incubated at final suspension density 1·7 mg. dry wt. of cells/ml. in buffered saline containing 0·75% (w/v) glucose and [^{14}C]phenylalanine with (condition 2) and without (condition 1) complete mixture of amino acids at final concentration of each component (other than phenylalanine) = 0·2 mg./ml.

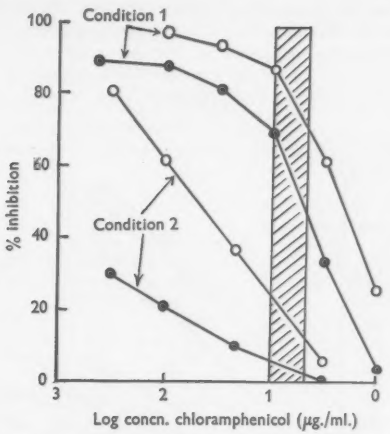


Fig. 2. Inhibition of incorporation of phenylalanine (○) and glutamic acid (●) by chloramphenicol in the presence (condition 2) and absence (condition 1) of a mixture of amino acids. Washed suspension of *Staph. aureus* incubated at final suspension density 1·7 mg. dry wt. of cells/ml. at 37° in buffered saline containing 0·75% (w/v) glucose and either 1·0 μmole [^{14}C]glutamic acid (specific activity 0·2 mc/m-mole) or 0·5 μmole [^{14}C]phenylalanine (specific activity 1·19 mc/m-mole)/ml. with (condition 2) and without (condition 1) amino acid mixture A (less either glutamic acid or phenylalanine, respectively) at a final concentration of each component = 0·2 mg./ml. Samples taken at 30 and 60 min., protein fraction prepared, dried and counted. Inhibition calculated as percentage rate of incorporation in the absence of chloramphenicol. Cross-hatched area shows range of antibiotic preventing growth of an inoculum of approx. 10^5 cells/ml. in fully nutrient medium.

dition 2 (0·45 μmole/ml.) is greater than that obtained under condition 1 (0·015 μmole/ml.). In the case of glutamic acid incorporation there was no difference in the values of the 'apparent Michaelis constant' under the two conditions (Gale & Folkes, 1953*c*).

Action of chloramphenicol on phenylalanine incorporation. Fig. 2 shows the effect of chloramphenicol on the incorporation of either phenylalanine or glutamic acid in the presence and absence of a complete amino acid mixture. It can be seen that the incorporation of phenylalanine is more sensitive to inhibition by chloramphenicol than is incorporation of glutamic acid whether other amino acids are present or not, and that both incorporations are more sensitive under condition 2 than under condition 1. Figs. 3*a* and 3*b* show the effect of two or three concentrations of chloramphenicol on the relation between phenylalanine concentration and rate of incorporation. Whether studied under condition 1 or 2, the lines obtained show a typical non-competitive inhibition of incorporation by chloramphenicol (see Lineweaver & Burk, 1934). Table 1 shows that there is a small increase in the calculated 'apparent Michaelis constant' when chloramphenicol inhibits phenylalanine incorporation under condition 1, whereas there is a marked decrease in the calculated value when inhibition occurs under condition 2. Similar experiments have been carried out with aureomycin and terramycin, and in these cases the inhibition is non-competitive and no change in the calculated Michaelis constants occurs, within experimental error, under condition 1 (see Fig. 3*a* and Table 1).

Action of p*-chlorophenylalanine on phenylalanine incorporation.* Figs. 3*a* and 3*b* also show the inhibitory action of two concentrations of *p*-chlorophenylalanine on phenylalanine incorporation under conditions 1 and 2. Under both conditions, the lines obtained are typically those given by competitive inhibition of incorporation (Lineweaver & Burk, 1934). Table 1 shows that there is a marked increase in the calculated 'Michaelis constants' under either condition 1 or 2 in the presence of *p*-chlorophenylalanine.

Action of p*-chlorophenylalanine on the incorporation of glutamic acid.* Table 2 shows that *p*-chlorophenylalanine has little inhibitory action on glutamic acid incorporation under either condition 1 or 2. In the experiments described so far, under condition 2 the only amino acid present in limiting concentration has been the labelled amino acid

whose incorporation is being studied. It seemed possible that the effect of both chloramphenicol and *p*-chlorophenylalanine on glutamic acid incorporation under condition 2 might be increased if the concentration of phenylalanine in the amino acid mixture were also made limiting. Table 2 shows that although the action of *p*-chlorophenylalanine is affected to some extent by the phenylalanine concentration, the analogue has little inhibitory action on glutamic acid incorporation under condition 2 even when the phenylalanine concentration is reduced to such a level that the incorporation of phenylalanine is almost completely abolished by the analogue. This conclusion was confirmed (Table 2, Expt. 4) by carrying out parallel experiments in which either the phenylalanine or the glutamic acid was labelled. Table 3 shows that the action of chloramphenicol on the incorporation of glutamic acid under condition 2 is not affected by the phenylalanine concentration.

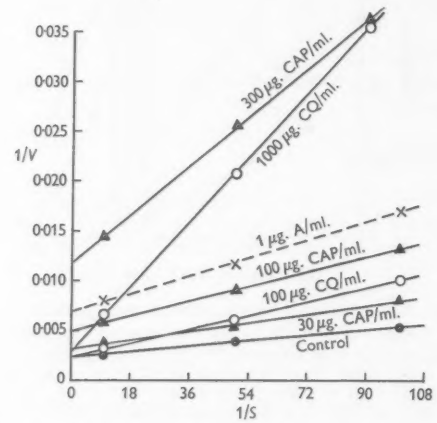


Fig. 3*a*. Effect of chloramphenicol (CAP), *p*-chlorophenylalanine (CQ) and aureomycin (A) on the incorporation of phenylalanine into the protein fraction of *Staphylococcus aureus* incubated with [^{14}C]phenylalanine and glucose. Details as for Fig. 1 (condition 1).

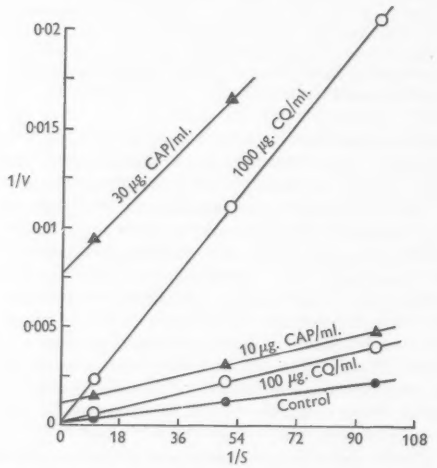


Fig. 3*b*. Effect of chloramphenicol (CAP) and *p*-chlorophenylalanine (CQ) on the incorporation of phenylalanine into the protein fraction of *Staphylococcus aureus* incubated with [^{14}C]phenylalanine, glucose and a complete mixture of amino acids. Details as for Fig. 1 (condition 2).

Relation between inhibition of protein synthesis and inhibition of incorporation. Experiments were carried out in parallel which enabled the action of *p*-chlorophenylalanine to be tested on net protein synthesis, determined as described by Gale & Folkes (1953*a*), and on the incorporation of either glutamic acid or phenylalanine. Table 4 shows that the inhibition of phenylalanine incorporation approximately parallels the inhibition of net protein synthesis, whereas the inhibition of glutamic acid incorporation bears no relation to that of net protein synthesis.

Table 1. *Effect of inhibitors on the 'apparent Michaelis constant' for the incorporation of phenylalanine into the protein fraction of* Staphylococcus aureus

(Washed suspension of *Staph. aureus* incubated at final suspension density 1·7 mg. dry wt. of cells/ml. for 1 hr. at 37° in buffered saline solution containing glucose 0·75% (w/v) and [^{14}C]phenylalanine (specific activity 1·19 mc/m-mole) at concentrations 0·01, 0·02 and 0·1 μmole/ml. with and without amino acid mixture A + glutamic acid at a final concentration of each component = 0·2 mg./ml. Inhibitors added as below. 'Apparent Michaelis constants' calculated according to Lineweaver & Burk (1934) in each case and expressed as μmole/ml.)

		Apparent Michaelis constant for phenylalanine incorporation	
Inhibitor	Concn. (μmole/ml.)	Amino acids present	Amino acids absent
None	—	0·45	0·012
Chloramphenicol	0·012	0·15	—
	0·04	0·07	—
	0·12	—	0·0165
	0·4	—	0·0205
	1·2	—	0·025
Terramycin	0·0064	—	0·012
	0·00064	0·22	—
Aureomycin	0·002	—	0·016
	0·00062	0·37	—
p-Chlorophenylalanine	5·0	0·54	0·091

Table 2. *Effect of* p*-chlorophenylalanine on the incorporation of phenylalanine or glutamic acid into the protein fraction of* Staphylococcus aureus

(Washed suspension of *Staph. aureus* incubated at final suspension density 1·7 mg. dry wt. of cells/ml. for 1 hr. at 37° in buffered saline solution containing 0·75% (w/v) glucose and unlabelled or ^{14}C-labelled glutamic acid (specific activity 0·9 mc/m-mole), and unlabelled or labelled [^{14}C]phenylalanine (specific activity 1·19 mc/m-mole) as below. Amino acid mixture is complete mixture A less glutamic acid and phenylalanine, added where shown at a final concentration of each component = 0·2 mg./ml. Results expressed as % inhibition of incorporation of radioactive amino acid occurring in absence of inhibitor.)

Expt.	Amino acid mixture	[^{12}C]-Glutamic acid (μmole/ml.)	[^{12}C]-Phenylalanine (μmole/ml.)	[^{14}C]-Glutamic acid (μmole/ml.)	[^{14}C]-Phenylalanine (μmole/ml.)	Radioactivity protein fraction no inhibitor (counts/min./mg.)	p-Chlorophenylalanine (μmole/ml.)	% Inhibition of incorporation of: [^{14}C]-Glutamic acid	% Inhibition of incorporation of: [^{14}C]-Phenylalanine
1	0	—	—	—	0·1	483	0·5	—	11
	0	—	—	—	0·1	483	5·0	—	57
	0	—	—	0·1	—	1816	5·0	0	—
2	+	1·3	—	—	0·2	1494	0·5	—	38
	+	1·3	—	—	0·2	1494	5·0	—	70
	+	1·3	—	—	0·1	858	5·0	—	88
3	+	—	13·0	0·1	—	2129	5·0	0	—
	+	—	1·3	0·1	—	2020	5·0	5	—
	+	—	0·13	0·1	—	2050	5·0	3	—
	+	—	0·013	0·1	—	2322	5·0	18	—
4	+	—	0·1	0·1	—	2000	5·0	4	—
	+	0·1	—	—	0·1	823	5·0	—	60

Table 3. *Effect of chloramphenicol on the incorporation of phenylalanine or glutamic acid into the protein fraction of* Staphylococcus aureus

(Details as for Table 2.)

Expt.	Amino acid mixture	[^{12}C]-Glutamic acid (μmole/ml.)	[^{12}C]-Phenylalanine (μmole/ml.)	[^{14}C]-Glutamic acid (μmole/ml.)	[^{14}C]-Phenylalanine (μmole/ml.)	Radioactivity protein fraction no inhibitor (counts/min./mg.)	Chloramphenicol (μmole/ml.)	% Inhibition of incorporation of: [^{14}C]-Glutamic acid	% Inhibition of incorporation of: [^{14}C]-Phenylalanine
1	0	—	—	—	0·3	622	0·4	—	62
	0	0·9	—	0·1	—	532	0·4	26	—
2	+	1·0	—	—	0·3	6653	0·04	—	88
	+	1·0	1·3	0·1	—	1603	0·04	36	—
3	+	0·9	13·0	0·1	—	1040	0·04	48·5	—
	+	0·9	1·3	0·1	—	1136	0·04	54·5	—
	+	0·9	0·13	0·1	—	1027	0·04	41·5	—
	+	0·9	0·013	0·1	—	1014	0·04	46·0	—
4	+	—	0·1	0·1	—	2000	0·015	4·0	—
	+	0·1	—	—	0·1	823	0·015	—	37

Actions of other antibiotics on phenylalanine incorporation. Table 5 shows the inhibition of phenylalanine incorporation under conditions 1 and 2 by a number of antibiotics. The inhibition of glutamic acid incorporation in parallel experiments is also given for comparison. The incorporation of phenylalanine, whether under condition 1 or 2, is more sensitive to the action of chloramphenicol, aureomycin or terramycin than is the incorporation of glutamic acid, but whereas the latter is sensitive to inhibition by penicillin or bacitracin under condition 1, these antibiotics have no effect on phenylalanine incorporation under the conditions used.

DISCUSSION

Woolley (1951) has suggested that the action of chloramphenicol is due to its relationship to phenylalanine. The incorporation of phenylalanine into the protein fraction of *Staph. aureus* is certainly more sensitive to inhibition by chloramphenicol than is the incorporation of glutamic acid but not more so than the incorporation of proline, leucine or tyrosine (Gale & Folkes, 1953*c*). Moreover, the difference in sensitivity is also shown with aureomycin and terramycin. Analysis of the nature of the inhibition

Table 4. *Action of* p*-chlorophenylalanine on net protein synthesis and on the incorporation of either phenylalanine or glutamic acid into the protein fraction of* Staphylococcus aureus

(Washed suspension of *Staph. aureus* incubated at final suspension density 2·0 mg. dry wt. of cells/ml. for 1 hr. at 37° with 0·75% (w/v) glucose and amino-acid mixture A less glutamic acid and phenylalanine (final concentration of each component = 0·2 mg./ml.) with additions of glutamic acid and phenylalanine as follows: (*a*) Unlabelled glutamic acid and phenylalanine; 100 mg. dry weight of cells; protein precipitated at end of incubation and in initial sample with 5% (w/v) cold trichloroacetic acid and protein-glutamate estimated after hydrolysis (Gale & Folkes, 1953*a*). (*b*) Unlabelled glutamic acid and ^{14}C-labelled phenylalanine (specific activity 1·19 mc/m-mole); 5·0 mg. dry weight of cells; protein fraction prepared and counted. (*c*) Unlabelled phenylalanine and ^{14}C-labelled glutamic acid (specific activity 0·1 mc/m-mole); otherwise as (*b*).)

Incubation mixture			Increase in protein-glutamate (mg./100 mg. dry wt. of cells)	Incorporation into protein fraction, no inhibitor (counts/min./5 mg. dry wt. of cells)		% Inhibition by 5·0 μmoles *p*-chlorophenylalanine/ml.		
Glucose amino acid mixture	Glutamic acid (μmole/ml.)	Phenyl-alanine (μmole/ml.)	(*a*)	[^{14}C]-Phenyl-alanine (*b*)	[^{14}C]-Glutamic acid (*c*)	(*a*)	(*b*)	(*c*)
+	1·0	0·01	0·17	672	909	100	89	20
+	1·0	0·1	0·92	5054	942	58	62	4
+	1·0	1·0	1·27	11805	915	2	18	2

Table 5. *Effect of antibiotics on the incorporation of glutamic acid or phenylalanine into the protein fraction of* Staphylococcus aureus

(Washed suspension of *Staph. aureus* incubated at final suspension density 1·7 mg. dry wt. of cells/ml. for 1 hr. at 37° in buffered saline containing 0·75% (w/v) glucose and either 1·0 μmole [^{14}C]glutamic acid (specific activity 0·2 mc/m-mole) or 0·1 μmole [^{14}C]phenylalanine (specific activity 1·19 mc/m-mole) with or without a complete amino acid mixture at final concentration of each component = 0·2 mg./ml. Results expressed as percentage rate of incorporation obtained in absence of inhibitor in each case.)

		% Inhibition of incorporation			
		Amino acid mixture present		Amino acid mixture absent	
Antibiotics	Concentration (μg./ml.)	Glutamic acid	Phenyl-alanine	Glutamic acid	Phenyl-alanine
Chloramphenicol	100	—	—	26	62
	10	70	88	6	21
Aureomycin	1	—	—	32	69
	0·3	51	85	—	—
Terramycin	3·0	—	—	34	55
	0·3	26	53	—	—
Penicillin	0·3	0	0	40	5
Bacitracin	100	0	0	55	0

of phenylalanine incorporation by chloramphenicol shows that it is non-competitive, and very different from the competitive inhibition shown by *p*-chlorophenylalanine. Clearly the action of chloramphenicol on protein synthesis cannot be ascribed to a specific inhibition of phenylalanine incorporation, although the fact that the 'apparent Michaelis constant' for the latter process shows some increase in the presence of chloramphenicol but not of aureomycin, may indicate that the structural relationship may influence the inhibitory action of the former antibiotic. Under condition 2 chloramphenicol produces a marked decrease in the 'apparent Michaelis constant' and this may be a reflection of the difference in the 'apparent Michaelis constants' under conditions 1 and 2, and of the fact that incorporation under condition 2 is more sensitive to chloramphenicol than under condition 1.

Under condition 2 the cells are able to accomplish a net synthesis of protein (Gale & Folkes, 1953*a*), and it might be thought that the incorporation of any labelled amino acid would provide a measure of protein synthesis under these conditions. If this were the case, an inhibitor which prevented protein synthesis should affect the incorporation of all

amino acids to the same extent. This is not the case; it has been shown above that chloramphenicol and *p*-chlorophenylalanine affect the incorporation of glutamic acid and phenylalanine (under condition 2) to different extents. In the case of *p*-chlorophenylalanine it is possible to inhibit the incorporation of phenylalanine almost completely without producing significant effect on the incorporation of glutamic acid, and the inhibition of protein synthesis correlates with the effect on phenylalanine. It would seem that inhibition of protein synthesis follows the inhibition of incorporation of that amino acid most sensitive to the inhibitor used. Prevention of protein synthesis by abolishing the uptake of phenylalanine has little or no effect on the rate of glutamic acid incorporation under condition 2, and this confirms the previous studies on glutamic acid incorporation (Gale & Folkes, 1953*c*) in which it was shown that addition of a complete amino acid mixture increased the rate of glutamic acid incorporation but that omission of phenylalanine from that mixture had little effect. It is clear that the rate of incorporation of a labelled amino acid into the protein fraction of a living cell cannot necessarily be taken as a measure of the rate of protein synthesis within that cell.

SUMMARY

1. Incubation of washed suspensions of *Staphylococcus aureus* with glucose and ^{14}C-labelled phenylalanine results in incorporation of the isotope into the protein fraction of the cells. The rate of incorporation can be markedly increased by the addition of a complete mixture of amino acids to the incubation mixture.

2. The incorporation of phenylalanine, whether in the presence or absence of the amino acid mixture, is competitively inhibited by *p*-chlorophenylalanine, and non-competitively inhibited by chloramphenicol, aureomycin or terramycin.

3. The incorporation of phenylalanine is more sensitive to the action of chloramphenicol, aureomycin or terramycin than is the incorporation of glutamic acid.

4. When a complete mixture of amino acids is present, an inhibitor may affect net protein synthesis, the incorporation of phenylalanine and the incorporation of glutamic acid to different extents. *p*-Chlorophenylalanine completely inhibits net protein synthesis and phenylalanine incorporation while being without significant effect on glutamic acid incorporation.

REFERENCES

Gale, E. F. & Folkes, J. P. (1953*a*). *Biochem. J.* **53**, 483.
Gale, E. F. & Folkes, J. P. (1953*b*). *Biochem. J.* **53**, 493.
Gale, E. F. & Folkes, J. P. (1953*c*). *Biochem. J.* **55**, 721.
Halvorson, H. O. & Spiegelman, S. (1952). *J. Bact.* **64**, 207.
Lineweaver, H. & Burk, B. (1934). *J. Amer. chem. Soc.* **56**, 658.
Woolley, D. W. (1951). *J. biol. Chem.* **185**, 293.

The Reaction of Fluorodinitrobenzene with the α- and ε-Amino Groups of Collagen

By JOANE H. BOWES and J. A. MOSS
British Leather Manufacturers' Research Association, Egham, Surrey

(*Received* 8 *January* 1953)

The technique developed by Sanger (1945) for the determination of free amino groups in proteins has been applied to collagen and procollagen with the object of gaining information about the form and size of the molecule. So far this technique has only been applied to a few of the insoluble proteins: wool keratin, myosin and tropomyosin. In keratin a comparatively large number of terminal amino groups were detected suggesting that the molecule consists of a relatively large number of polypeptide chains (Middlebrook, 1951; Blackburn, 1949). With myosin and tropomyosin, however, Bailey (1951) was unable to detect the presence of any terminal amino groups and suggested the possibility of a cyclic molecule.

The evidence available on the structure of collagen from X-ray and electron microscope observations has recently been reviewed by Bear (1952). The fibrils are considered to be formed of regularly coiled polypeptide chains arranged roughly parallel to one another, with regions of similar chemical structure matching transversely. Collagen contains no cystine and there is no evidence for any covalent cross-linkages between the chains, suggesting that, in contrast to keratin, the molecule may consist of a single polypeptide chain. Pauling & Corey (1951),

however, have proposed a structure in which the molecule is considered to be formed of three polypeptide chains, each coiled into a helix and joined to one another by hydrogen bonds.

Porter (1948) has found that, with certain albumins and globulins, not all the ϵ-amino groups react with 1-fluoro-2:4-dinitrobenzene (FDNB) unless the protein is first denatured. With a fibrous protein such as collagen any steric factors affecting the reactivity of such groups are likely to be more pronounced and the non-reactivity may also extend to terminal α-amino groups.

The effect of various treatments on the reactivity of the ϵ-amino groups in collagen towards FDNB and on the liberation of α-amino groups has, therefore, also been considered.

A preliminary account of the work described in this paper was given at the Second International Congress of Biochemistry, Paris, 1952.

MATERIALS

Collagen was prepared from the middle layer of ox hide as described by Bowes & Kenten (1948*a*).

Procollagen was prepared from fresh calf skin as described by Orekhovich, Tustanovskiĭ, Orekhovich & Plotinkova (1948). The calf skin was shaved, cut into small pieces and disintegrated in a large Wiley mill (A. Thomas Co., Philadelphia, U.S.A.). The mill had to be stopped at frequent intervals and the macerated material removed by hand. The macerate (1800 g.) was extracted twice with 10 l. 0·1 M sodium phosphate buffer (pH 8·6) to remove albumins and globulins, and then three times with 10 l. 0·12 M sodium citrate buffer (pH 3·62). All extractions were carried out at 4°. The procollagen was precipitated from the citrate extracts by the addition of NaCl to a final concentration of 5% (w/v). The procollagen was purified by dissolving in slightly acidified water (pH 4·0), and dialysing against tap water.

Modification of collagen

Before treatment the collagen was cut into small pieces, of area about 1 cm.^{2}, or ground in a laboratory-model Wiley mill to pass a 40-mesh/in. sieve.

Heat shrinkage. Pieces of collagen were heated in water at 65–68° for 15 min., cooled and dehydrated with acetone.

Urea treatment. Powdered collagen was immersed in 8 M urea for 24 hr. at laboratory temperature.

Solution in formic acid. Powdered collagen (9·0 g.) was suspended in 500 ml. anhydrous formic acid at 40°. The collagen swelled, and after 18 hr. the greater part had dissolved. The solution was filtered with aid of Celite 545 and Hyflo Super-Cel (Johns-Manville Co., London) and the collagen precipitated by neutralization with 40% (w/v) NaOH. During precipitation the solution was cooled in ice and stirred continuously. The fibrous precipitate was separated and washed with 25% (v/v) aqueous ethanol. The anhydrous formic acid was prepared from the A.R. acid by drying over copper sulphate and distilling under reduced pressure (Garner, Saxton & Parker, 1911).

Alkaline treatment. Pieces of collagen about 0·5 cm.^{2} were immersed in a solution of NaOH and $Ca(OH)_2$ at pH 13·0 for 14 days at 20° (Bowes & Kenten, 1948*b*). Such treatment has been found to dissolve about 5% of the collagen, some as large peptides and some as amino acids and small peptides (Bowes & Kenten, 1948*b*).

Treatment with hyaluronidase. Powdered collagen (5 g.) was treated with half a 1000 unit ampoule of Hyalase (Bengers, Holmes Chapel, Cheshire) dissolved in 150 ml. 0·33 M sodium citrate buffer (pH 4·6) for 16 hr. at 37°. Another 5 g. sample of collagen was treated with heat-inactivated Hyalase under the same conditions. Hyalase is prepared from mammalian testes and may, therefore, be expected to act on chondroitin sulphate as well as hyaluronic acid (Humphrey, 1946; Mathews, Roseman & Dorfman, 1951).

After the enzyme treatment, the collagen was washed by decantation with two 100 ml. portions of the citrate buffer for 8 and 16 hr., followed by two 100 ml. portions of distilled water for similar periods. It was then filtered off, dehydrated with acetone and air-dried.

METHODS

Treatment with fluoro-2:4-dinitrobenzene

Amounts of powdered collagen varying from 1 to 10 g. were treated with FDNB as described by Sanger (1945). For example, 10 g. collagen, suspended in 75 ml. sat. aqueous $NaHCO_3$ and 150 ml. ethanol, were treated with 8 ml. FDNB in a stoppered flask at room temperature. The time of treatment was usually 3 days, but in some cases was extended to 2 or 3 weeks. At the end of the treatment the reaction mixture was decanted off and the collagen thoroughly washed, first with water, then with ethanol and finally with ether. It was then air-dried.

Removal of soluble yellow material, presumably dinitrophenols, etc., by washing with ethanol was very slow, and in later experiments the DNP-protein was Soxhlet-extracted with ethanol for 16 hr. This was found to remove dinitrophenols almost completely.

The dinitrophenyl (DNP) derivatives of gelatin and formic acid-treated collagen were soluble in water and were not readily precipitated by ethanol or acetone. The reaction mixture was neutralized, extracted with ether, and the aqueous layer saturated with $(NH_4)_2SO_4$. The gummy precipitates formed were dialysed first against tap water, and then against distilled water for 3 days, and dried *in vacuo* at room temperature.

Hydrolysis, separation and identification of DNP-amino acids

Two methods of hydrolysis were used: (1) 12 hr. with boiling 6 N-HCl under reflux; (2) 16 hr. in a sealed tube with 10 N-HCl at 105°. Both gave essentially similar results. The latter method was preferred and used for the majority of the experiments since it causes less decomposition of DNP-proline and DNP-hydroxyproline. These residues were considered to be possible end groups in collagen. For the separation of α-DNP-amino acids, 0·5–1 g. of the DNP-protein was usually taken for hydrolysis. Up to 5 g. were taken in attempts to detect a terminal residue in collagen. The hydrolysate was extracted with ether and the α-DNP-amino acids separated on buffered silica gel columns similar to those described by Middlebrook (1949, 1951). The ether extract was first run on a column buffered to pH 6·66

with phosphate buffer (54·6 g. NaH_2PO_4, $2H_2O$ + 53·7 g. Na_2HPO_4, $12H_2O$/l.) and developed with ether saturated with the same buffer solution; this separated glutamic and aspartic acids, serine, threonine and glycine. The faster-moving DNP-amino acids were then separated on a column buffered with the pH 6·66 phosphate buffer and developed with $CHCl_3$ containing 3% (v/v) butanol and saturated with the buffer solution. Additional columns, as described by Sanger (1945) and Blackburn (1949), were used for further separations and confirmation.

For the identification and determination of ε-DNP-lysine and ε-DNP-hydroxylysine smaller amounts (approx. 10–20 mg. of the DNP-protein) were hydrolysed, the hydrolysates were taken to dryness and run on unbuffered silica-gel columns using 66% (v/v) ethyl methyl ketone in ether, or 30% (v/v) *n*-butanol in $CHCl_3$ (Sanger, 1945). In later experiments Hyflo Super-Cel was substituted for silica gel.

The identity of the bands observed was checked by running with authentic DNP derivatives, by paper chromatography of the DNP-amino acids, and of the free amino acids following hydrolysis with saturated $Ba(OH)_2$ for 1 hr. at 105° (Mills, 1950).

The most satisfactory system for the development of paper chromatograms of the DNP-amino acids was found to be *n*-amyl alcohol shaken with an equal volume of 2N ammonia solution, the organic phase being used for development and the aqueous phase for saturation of the cabinet.

Recovery of DNP-amino acids

The recovery of DNP-glycine, DNP-proline and ε-DNP-lysine when added to collagen and hydrolysed in a sealed tube for 16 hr. with 10N-HCl at 105° were 50, 20 and 80%, respectively. The values for DNP-lysine and DNP-glycine were approximately the same as those obtained by Porter & Sanger (1948) under the same conditions, and their values were used in making corrections for the decomposition of the remainder of the DNP-amino acids.

After hydrolysis for 64 hr. only 38% of ε-DNP-lysine added to collagen could be recovered.

Determination of DNP-amino acids

The α-DNP-amino acids were determined colorimetrically in 1% (w/v) $NaHCO_3$ solutions and ε-DNP-lysine in 0·75N-HCl in 25% (v/v) aqueous ethanol.

The amount of DNP-amino acid derived from a given weight of the original collagen was calculated from the weight of the DNP-collagen taken for hydrolysis, corrected for moisture and ash content, for dinitroaniline present, and for DNP groups attached to ε-amino groups. DNP groups attached to terminal amino groups were small in number in all cases, and were neglected. The appropriate corrections were made for decomposition of the DNP-amino acids during hydrolysis.

Determination of lysine and free amino nitrogen

Lysine was determined colorimetrically with ninhydrin following chromatographic separation on an ion-exchange resin (Moore & Stein, 1951).

Free amino nitrogen on the powdered DNP-protein was determined by the Van Slyke procedure using a modified reaction chamber as described by Doherty & Ogg (1943).

RESULTS

Reactivity of ε-amino groups

Both ε-DNP-lysine and ε-DNP-hydroxylysine were identified in hydrolysates of DNP-collagen and DNP-procollagen. The amount of ε-DNP-lysine obtained from DNP-collagen was only equivalent to 17 m-moles/100 g. protein, representing only about half the lysine content. Prolonging the time of reaction with FDNB to 14 or 21 days did not increase the amounts of ε-DNP-lysine found. With procollagen a rather higher proportion of the lysine was recovered as the DNP derivative. ε-DNP-hydroxylysine was not determined quantitatively, but the intensity of the yellow band due to this derivative suggested that the amount present was only equivalent to about half the hydroxylysine content of the collagen.

Following the observation of Porter (1948) that the ε-amino groups of some soluble proteins do not all react with FDNB unless the protein is first denatured, the possibility that some of the ε-amino groups of collagen are also unreactive was investigated. Treatment in 8M urea solution, in alkali, heating in water to 68° or dissolving in formic acid and reprecipitating did not increase the amounts of ε-DNP-lysine found after reaction with FDNB, and there was some suggestion that heat shrinkage actually caused a slight decrease (Table 1). Even with gelatin there was no increase in the amount of ε-DNP-lysine found.

Only small amounts of nitrogen were evolved from all the DNP-proteins by the action of nitrous acid in the usual Van Slyke procedure, and direct determination of lysine in DNP-substituted preparations of collagen and hyaluronidase-treated collagen gave values of only 4 and 3 m-moles/100 g. respectively. These observations suggested that failure to recover all the lysine as the ε-DNP derivative was not primarily due to non-reactivity of a proportion of the lysine to FDNB.

The possibility that the remaining lysine was present as α-DNP-lysine, αε-diDNP-lysine or as resistant DNP-lysine peptides was next considered. No evidence for the presence of these was obtained from the columns, the only unidentified band being a dirtyish yellow one running very slowly on the ether-phosphate buffer column, considered to be a decomposition product of DNP derivatives. Unless a large number of different peptides was formed, relatively large amounts would have to be present to account for 10 m-moles lysine/100 g. protein. Ether-soluble DNP-peptides would, therefore, almost certainly have been detected since, in some instances, the equivalent of 2–3 g. hydrolysed protein was run on the columns. Much smaller amounts were used for the examination of the aqueous extract owing to the large amount of

ε-DNP-lysine present, and small amounts of water-soluble DNP-peptides might not have been detected. Increase in the time of hydrolysis in a sealed tube with 10N hydrochloric acid at 105° from 16 to 64 hr. caused only a slight increase in the ε-DNP-lysine found (Table 2). Owing to the extensive decomposition of ε-DNP-lysine which occurred during this longer time of hydrolysis, and to the large correction factor which had to be applied, this slight increase was not considered significant.

Terminal amino groups

In addition to yellow bands due to dinitrophenols and dinitroaniline (identified by m.p.) and DNP-amino acids, there was a dirty-yellow material which stayed at the top of the ether-phosphate buffer columns, and a pinkish band running fast on all columns. The first slow band was probably due to some decomposition product of DNP-amino acids, while the fast band which was obtained with hydro-

Table 1. *Free amino groups of collagen and modified collagens*

	α-Amino groups		ε-DNP-lysine found	
	(m-moles/100 g.)	Mol.wt. containing one residue	(m-moles/100 g.)	(% of total lysine residues)
Collagen*	None detected		17	55
Urea-treated				
Aspartic acid	0·07	1 400 000	18	59
Heat-shrunk				
Aspartic acid	0·15	700 000	15	48
Glutamic acid	0·03	3 300 000		
Alkali-treated				
Aspartic acid	0·07	1 400 000	17	55
Glutamic acid	0·17	600 000		
Glycine	0·42	250 000		
Phenylalanine	0·04	2 500 000		
Dissolved in formic acid				
Aspartic acid	0·43	250 000	17	55
Glutamic acid	0·15	700 000		
Glycine	0·65	150 000		
Alanine	0·30	350 000		
Treated with hyaluronidase				
Aspartic acid	0·09	1 100 000	17	55
Alanine	0·16	600 000		
Glycine	0·12	800 000		
Threonine	0·03	3 300 000		
Gelatin (commercial)				
Aspartic acid	0·28	350 000	17	55
Glutamic acid	0·12	830 000		
Glycine	0·57	180 000		
Threonine	0·14	710 000		
Procollagen†				
Aspartic acid	0·06	1 650 000	17	68
Alanine	0·04	2 500 000		

* Lysine content of collagen, 31 m-moles/100 g. † Lysine content of procollagen, 25 m-moles/100 g.

Table 2. *Recovery of ε-DNP-lysine from DNP-proteins*

		m-moles/100 g. DNP-protein			
DNP-protein	Time of hydrolysis* (hr.)	ε-DNP-lysine†	Lysine‡	Free NH_2-N (Van Slyke)	Lysine not accounted for
Collagen	16	17	4	5	10
	16	17	3	—	11
Alkali-treated collagen	16	17	—	—	—
	64	19	—	—	—

* Hydrolysis in a sealed tube with 10N-HCl at 105°.
† Corrected for decomposition. Recovery of ε-DNP-lysine added to collagen and hydrolysed under the same conditions was 80% and 38% after 16 and 64 hr. respectively.
‡ By method of Moore & Stein (1951).

lysates of collagen as well as DNP-collagen was considered to be due to the protein itself and not to any reaction product of FDNB. The eluate corresponding to this band had a smell of charred sugar.

Collagen. No α-DNP-amino acids were detected in hydrolysates of collagen treated with FDNB, even when amounts equivalent to 2–3 g. protein were placed on the columns. It was estimated that using these amounts most amino acids would be detected if present as a terminal group to the extent of one residue/mol.wt. 4 or 5 million. Recovery tests indicated that DNP-proline, which is the DNP-amino acid least stable to hydrolysis, would have been detected if proline were present as a terminal residue to the extent of one residue/mol.wt. 1 500 000.

Procollagen. Small amounts of DNP-aspartic acid and DNP-alanine were found in hydrolysates of DNP-procollagen. It is possible that these small amounts arise from extraneous matter and do not represent terminal amino groups.

Modified collagens. Small amounts of DNP-amino acids were detected in the hydrolysates of the DNP derivatives of the modified collagens and gelatin (see Table 1). Owing to the number of correction factors involved and the small amounts concerned, the values given in Table 1 can only be considered to be correct to within ±20% of the true value.

DNP-Aspartic acid was found in hydrolysates of the DNP derivatives of all the modified collagens and gelatin. With urea-treated collagen this was the only DNP-amino acid found, with heat-shrunk collagen very small amounts of DNP-glutamic acid were also found, and with alkali-treated collagen DNP-glutamic acid, glycine and phenylalanine.

Alanine appeared as an end group when the modification of the collagen involved treatment with acid or slightly acid solutions, e.g. solution in formic acid or extraction with a citrate buffer at pH 4·0 as with procollagen.

The collagen dissolved in formic acid and the commercial gelatin behaved similarly, the DNP derivatives were soluble and the DNP-amino acids found were similar, except that DNP-alanine was found in the formic acid-treated collagen and DNP-threonine in gelatin.

After treatment with hyaluronidase, aspartic acid, alanine, glycine and a trace of threonine were found as terminal residues. No free α-amino groups were found in the collagen treated with the heat-inactivated enzyme under the same conditions, and it seems unlikely that the terminal residues found could have arisen from the hyaluronidase itself. It also seems unlikely that the liberation of α-amino groups from such a variety of amino acids can all be due to removal of polysaccharide actually combined with these groups. It is possible that some hydrolysis may have taken place during the treatment, breakdown of polysaccharide having increased the susceptibility of the peptide bonds to hydrolysis at 37°. In this connexion Jackson (1952) reports that treatment of tendon collagen with hyaluronidase increases its solubility in dilute acetic acid.

DISCUSSION

In studying the reactions of a fibrous protein such as collagen it is always difficult to assess whether the reaction has gone to completion or not. It was hoped that combination of FDNB with the ε-amino groups of lysine could be used as an indication of the course of the reaction. It was found, however, that, although all the ε-amino groups are free to react with nitrous acid (Bowes & Kenten, 1948*a*), ε-DNP-lysine equivalent to only about half these groups was recovered from the DNP-proteins. It was at first thought that this was due to non-reactivity of a proportion of the ε-amino groups towards FDNB as observed by Porter (1948) and others. However, the presence of only small amounts of free lysine in the hydrolysates of DNP-collagen, and the small amounts of nitrogen evolved by nitrous acid in the Van Slyke apparatus indicated that, while it was possible that a few of the lysine groups were unreactive, this could not be the only explanation of the low recovery of ε-DNP-lysine. Out of 31 m-moles lysine/100 g. collagen, approx. 17 were recovered as ε-DNP-lysine (corrected for decomposition during hydrolysis) and 3–4 m-moles were found as unchanged lysine, leaving 10–11 m-moles to be accounted for (Table 2). No evidence was obtained of the presence of α-DNP-lysine, αε-diDNP-lysine or of DNP-lysine peptides in the hydrolysates; increasing the time of hydrolysis did not significantly increase the amount of ε-DNP-lysine found and no yellow bands which might be due to these lysine derivatives were observed on any of the silica-gel columns. There remains the possibility that ε-DNP-lysine is much less stable to acid when combined in the collagen than when present as the free amino acid. In other proteins studied there has been no indication of such decomposition, reasonable agreement being found between the ε-DNP-lysine and the total lysine content of the protein, provided this has been denatured before treatment with FDNB (Sanger, 1952). If extensive decomposition of ε-DNP-lysine does occur, therefore, it would appear to be peculiar to collagen. The possibility that this also applies to other DNP-amino acids should be particularly borne in mind when considering the results of end-group determinations on collagen and modified collagen.

It is interesting to speculate on possible reasons for failure to detect terminal α-amino groups in

collagen. First, the molecule may be very large. Assuming that decomposition of DNP-amino acids during the hydrolytic treatment is no greater when in peptide combination than when free, it was calculated that most terminal amino acid residues would have been detected if present to the extent of one residue/mol.wt. 4 or 5 million. Even if more extensive decomposition occurred it should still have been possible to detect any terminal residue, with the possible exception of proline, if present to the extent of one residue/mol.wt. 1 to 2 million. The molecular weight may, therefore, be of the order of 1 to 2 million at least and probably considerably higher. Average molecular weights of up to 250000 have been reported for gelatin (Pouradier & Venet, 1950), and as some peptide bonds are almost certainly broken during the conversion of collagen into gelatin it is quite possible that the molecular weight of collagen is several times this figure.

The molecule may be cyclic; this does not at first seem to fit in with the conception of a fibrous protein. There is some indication, however, that end groups may be absent in other fibrous proteins, and Bailey (1951), who was unable to detect any end groups in myosin and tropomyosin, has suggested, on the basis of measurements of molecular size (Tsao, Bailey & Adair, 1951), that the tropomyosin molecule is cyclic.

Finally, there is the possibility that the terminal amino groups are inaccessible or masked in some way. The presence of aspartic acid as a terminal residue in collagen which had received such relatively mild treatments as immersion in urea solution or heating to 68° suggests that it may be a terminal residue inaccessible to FDNB in the original collagen. On the other hand, there is evidence that aspartic acid is readily liberated from proteins. Partridge & Davis (1950) and Adair, Partridge & Davis (1951) have shown that aspartic and glutamic acids are preferentially liberated in the partial hydrolysis of a number of proteins with weak acids, and Blackburn (1950*a*, *b*) finds that this also occurs when wool is partially hydrolysed with dilute hydrochloric acid. The free α-amino groups of aspartic acid may, therefore, arise from hydrolysis of peptide bonds during the treatment. In view of the importance now ascribed to mucopolysaccharides in the formation of collagen, the possibility of masking of terminal groups by association with chondroitin sulphate or other polysaccharide is worth consideration. Unfortunately the results of the experiment with testicular hyaluronidase were inconclusive, as it is uncertain whether all the amino groups liberated by this treatment arose from removal of polysaccharide. It may be significant, however, that two of the terminal residues found, aspartic acid and alanine, were also found in procollagen.

There is no evidence of any co-ordinate links in collagen, and it is fairly generally agreed that collagen owes its stability to a large number of interchain hydrogen bonds. Several considerations favour the conception of a molecule consisting of a single long polypeptide chain, and the absence of detectable terminal amino groups is most readily explained on the basis of a very large molecule or the masking of this group, most probably aspartic acid, by association with mucopolysaccharide or some other group. If aspartic acid is not a terminal residue, then the peptide bond involving this amino acid is very labile.

A number of other end groups, in addition to aspartic acid, were found in the modified collagens; these probably arise from hydrolysis of peptide bonds, suggesting that certain peptide bonds involving the amino groups of glutamic acid, glycine, alanine and to a lesser extent threonine and phenylalanine are particularly labile. There is, however, the possibility that some of these DNP-amino acids arise from the uncovering of α-amino groups by the splitting off of DNP-aspartic acid during the treatment with FDNB. This has been found to occur with 'old yellow enzyme' (Weygand & Junk, 1951).

The detection of an appreciable number of free α-amino groups in collagen which has been dissolved in formic acid and reprecipitated calls for some comment. It is often assumed that collagen can be recovered unchanged from formic acid solution, but the present results suggest that the product obtained more nearly resembles gelatin than the original collagen, the number of free α-amino groups actually exceeding those of a commercial gelatin.

SUMMARY

1. The reaction of fluorodinitrobenzene with the amino groups of collagen, modified collagen and procollagen has been studied.

2. Only 55–60% of the lysine could be recovered as the ε-dinitrophenyl (DNP) derivative from collagen, modified collagen or gelatin which had been treated with fluorodinitrobenzene. Only small amounts of free amino nitrogen and unchanged lysine were found in the DNP proteins leaving approx. 30% of the lysine unaccounted for. The possibility of decomposition of ε-DNP-lysine during hydrolysis, and of the presence of acid-resistant ε-DNP-lysine peptides is considered.

3. No terminal residues were detected in collagen, and possible reasons for this are discussed.

4. Small amounts of various end groups were found in procollagen, the modified collagens, and gelatin. Aspartic acid was most frequently found as a terminal residue, followed by glutamic acid,

glycine, alanine and threonine. With the possible exception of aspartic acid these are considered to arise from hydrolysis of peptide bonds during the treatments. Aspartic acid is either a terminal residue which is inaccessible to fluorodinitrobenzene or the peptide bond involving its α-amino group is especially labile.

Thanks are due to the Council of the British Leather Manufacturers' Research Association for permission to publish this paper.

REFERENCES

Adair, G. S., Partridge, S. M. & Davis, H. F. (1951). *Nature, Lond.*, **167**, 605.
Bailey, K. (1951). *Biochem. J.* **49**, 23.
Bear, R. S. (1952). *Advanc. in Protein Chem.* **7**, 69.
Blackburn, S. (1949). *Biochem. J.* **45**, 579.
Blackburn, S. (1950*a*). *Biochem. J.* **47**, 443.
Blackburn, S. (1950*b*). *Biochem. J.* **47**, xxviii.
Bowes, J. H. & Kenten, R. H. (1948*a*). *Biochem. J.* **43**, 358.
Bowes, J. H. & Kenten, R. H. (1948*b*). *Biochem. J.* **43**, 365.
Doherty, D. G. & Ogg, C. L. (1943). *Industr. Engng Chem. (Anal.)*, **15**, 75.
Garner, J. B., Saxton, B. & Parker, H. O. (1911). *Amer. chem. J.* **46**, 236.
Humphrey, J. H. (1946). *Biochem. J.* **40**, 442.
Jackson, D. S. (1952). *Biochem. J.* **54**, 638.
Mathews, M. B., Roseman, S. & Dorfman, A. (1951). *J. biol. Chem.* **188**, 327.
Middlebrook, W. R. (1949). *Nature, Lond.*, **164**, 501.
Middlebrook, W. R. (1951). *Biochim. biophys. Acta*, **7**, 547.
Mills, G. L. (1950). *Nature, Lond.*, **165**, 403.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Orekhovich, V. N., Tustanovskiĭ, A. A., Orekhovich, K. D. & Plotinkova, N. E. (1948). *Biochemistry, Leningr.*, **13**, 55.
Partridge, S. M. & Davis, H. F. (1950). *Nature, Lond.*, **165**, 62.
Pauling, L. & Corey, R. B. (1951). *Proc. nat. Acad. Sci., Wash.*, **37**, 272.
Porter, R. R. (1948). *Biochim. biophys. Acta*, **2**, 105.
Porter, R. R. & Sanger, F. (1948). *Biochem. J.* **42**, 287.
Pouradier, J. & Venet, A. M. (1950). *J. Chim. phys.* **47**, 11.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Sanger, F. (1952). *Advanc. in Protein Chem.* **7**, 1.
Tsao, T. C., Bailey, K. & Adair, G. S. (1951). *Biochem. J.* **49**, 27.
Weygand, F. & Junk, R. (1951). *Naturwissenschaften*, **38**, 433.

The Catalytic Effect of Molybdate on the Hydrolysis of Organic Phosphates

By H. WEIL-MALHERBE
Research Department, Runwell Hospital, Wickford, Essex

(*Received* 16 *May* 1953)

Lutwak & Sacks (1953) recently investigated the effect of molybdate on the hydrolysis of organic phosphates at a temperature of 100°. Two series of experiments were carried out, one at a pH of about 5·5 and the other in N sulphuric acid. The inorganic phosphate formed after certain time intervals was determined by two methods: (*a*) by direct colorimetric estimation according to Fiske & Subbarow (1925), and (*b*) by precipitation with magnesia mixture, followed by a colorimetric analysis of the dissolved precipitate. Both methods were in agreement when samples were analysed, which did not contain molybdate during heating; on the other hand, when organic phosphates were heated together with molybdate, the inorganic phosphate estimated by the direct colorimetric method was considerably in excess of that estimated after precipitation with magnesia mixture; it was, moreover, often in excess of the theoretical value for complete hydrolysis, in one case by about 400%. These results were interpreted as indicating the formation of organophosphomolybdate complexes, not precipitable by magnesia mixture, but reducible by the Fiske-Subbarow reagent. If this interpretation were correct, it would cast doubt on the significance of some of the results obtained earlier by Weil-Malherbe & Green (1952) in an investigation in which the molybdate effect was studied under different experimental conditions and with a different analytical technique. It was therefore of interest to find out (*a*) whether the effect of Lutwak & Sacks would be observed with their analytical techniques under the milder experimental conditions that were used in most of the experiments of Weil-Malherbe & Green, i.e. at a temperature of 80° or less and in the pH range between 3 and 6; and (*b*) whether it could be observed under the more rigorous conditions of Lutwak & Sacks with the analytical technique of Weil-Malherbe & Green who used a stannous chloride method of phosphate

estimation (Martin & Doty, 1949). The hydrolysis of three of the compounds used in the American investigation was therefore determined under the following conditions: (i) at pH 5·5 and 80°, (ii) in N acid at 100°, and by the following methods: (*a*) that of Martin & Doty (1949) using stannous chloride as reducing agent, (*b*) that of Fiske & Subbarow (1925) using aminonaphthol sulphonic acid and (*c*) after precipitation with magnesia mixture.

EXPERIMENTAL

The test solutions contained 1% ammonium molybdate and either 0·05M sodium acetate-acetic acid buffer, pH 5·5, or N-H_2SO_4. The substrate concentration was 1·6 mM. The substrates used were: fructose 6-phosphate, adenosine 5′-phosphate and sodium phenyl phosphate. Samples for the estimation of inorganic phosphate were withdrawn at zero time and after 2 and 4 hr.

Analytical methods. The modification of the method of Martin & Doty (1949) used in this laboratory has been described (Weil-Malherbe & Green, 1952). We now use the *iso*butanol-benzene mixture as recommended by Martin & Doty for the extraction; it produces a better separation of the phases, especially after perchloric acid digestion. A 4 ml. sample of the extract was withdrawn, instead of 5 ml. which were sometimes difficult to obtain, and was mixed with 4 ml. acid ethanol and 0·2 ml. of dilute $SnCl_2$ solution.

The method of Fiske & Subbarow was used in the modification of Umbreit, Burris & Stauffer (1945).

Precipitation with magnesia mixture was carried out on a 20 ml. sample. After the solution had been made alkaline to phenolphthalein with NH_3, a further 2 ml. of conc. NH_3 soln. (sp.gr. 0·880) and 2 ml. of magnesia mixture (5 g. $MgCl_2$, $6H_2O$, 10 g. NH_4Cl and 10 ml. conc. NH_3 soln. made up to 100 ml. with water) were added. The solution was left at 3° for 18 hr. After washing with 2% NH_3 soln. the precipitate was dissolved in acid and estimated by the method of Martin & Doty.

RESULTS

Comparison of phosphate methods

All three methods of phosphate estimation were in agreement in the experiments carried out at pH 5·5 and 80°, whether molybdate was present or not (Table 1). The differences observed may be ascribed to analytical errors; they do not substantiate the existence of the effect under these conditions. No blue colour developed in the samples heated with molybdate.

In the experiments performed at 100° in N acid agreement between the three methods of estimation was again reasonable when molybdate was absent. On the other hand, with fructose 6-phosphate or adenosine 5′-phosphate as substrate and with molybdate present, the three methods gave strikingly different results. Those obtained with the method of Fiske & Subbarow greatly exceeded the theoretical value for complete hydrolysis. This was not the case when the method of Martin & Doty was used, but even then the results were higher than those obtained after precipitation with magnesia mixture (Table 1).

An intense-blue colour developed in these samples during the exposure to heat. With phenyl phosphate as substrate the solution turned bright green, presumably owing to the admixture of some blue colour to a yellow background of phosphomolybdic acid. Here the effect of Lutwak & Sacks was much less in evidence. Again 'phosphate by precipitation' agreed better with 'phosphate by Martin & Doty' than with 'phosphate by Fiske & Subbarow'.

As to the catalytic effect of molybdate at pH 5·5 and 80°, it was absent with phenyl phosphate, slight

Table 1. *Hydrolysis of organic phosphates*

(M. & D., method of Martin & Doty (1949); F. & S., method of Fiske & Subbarow (1925); Mg, precipitation with magnesia mixture.)

Compound	Conditions	Time (hr.)	Without molybdate			With molybdate			Blue colour
			M. & D.	F. & S.	Mg	M. & D.	F. & S.	Mg	
			Hydrolysis (%)						
Sodium phenyl phosphate	80°, pH 5·5	2	14·8	13·0	13·9	14·1	15·0	14·6	–
		4	26·3	25·4	25·6	28·3	27·0	25·9	–
	100°, N-H_2SO_4	2	29·8	30·2	26·9	49·5	56·7	41·6	+
		4	55·8	53·9	53·9	72·7	83·6	67·5	+
Fructose 6-phosphate	80°, pH 5·5	2	5·1	4·5	4·8	23·6	24·1	24·0	–
		4	10·2	9·8	10·0	36·0	36·0	35·8	–
	100°, N-H_2SO_4	2	36·2	32·2	32·9	61·7	333	37·9	+ + +
		4	57·8	59·2	54·5	71·4	364	56·5	+ + +
Adenosine 5′-phosphate	80°, pH 5·5	2	0·9	1·2	0·9	1·8	2·6	1·7	–
		4	1·8	1·8	1·8	3·4	3·4	3·2	–
	100°, N-H_2SO_4	2	14·8	13·6	12·7	80	419	32	+ + +
		4	30·9	30·7	27·3	89	458	36	+ + +

Table 2. *Recovery of inorganic phosphate*

(Final concns. KH_2PO_4, 1·6 mM; fructose, 3·33 mM; H_2SO_4, 1 N. Temp. 100°. M. & D., method of Martin & Doty (1949); F. & S., method of Fiske & Subbarow (1925); Mg, precipitation with magnesia mixture.)

Time (hr.)	Without molybdate			With 1% ammonium molybdate			
	M. & D.	F. & S.	Mg	M. & D.	F. & S.	Mg	Blue colour
	Recovery (%)						
0	100	102·8	95·2	100	101·5	89·5	–
2	101·5	102·0	93·1	150	595	54·5	+ + +
4	104·1	103·9	96·2	168	700	54·5	+ + +

with adenosine 5′-phosphate and strong with fructose 6-phosphate. The last result confirms previous observations (Weil-Malherbe & Green, 1952). At pH 0 and 100° the catalytic effect of molybdate was evident in the case of phenyl phosphate, but cannot be assessed in the experiments with the other two substrates.

Recovery experiments

It was concluded from these results that the effect of Lutwak & Sacks is connected with the formation of blue colour during the exposure to heat. The recovery of inorganic phosphate was therefore studied under conditions in which the formation of molybdenum blue and phosphomolybdenum blue could be expected. Fructose (0·06%) was used as reducing agent, so as to approximate the conditions of the experiment to those in which the hydrolysis of fructose 6-phosphate was studied. The experiment included two controls, one in which molybdate, and one in which phosphate was absent.

The results are shown in Table 2. Again, the samples containing molybdate developed an intense-blue colour during heating, not only in the presence of phosphate, but also in its absence. Recoveries in absence of molybdate were quantitative, the slight increase found with the colorimetric methods probably being due to evaporation losses. In presence of molybdate extremely high values were obtained with the Fiske & Subarrow method. With the method of Martin & Doty, too, erroneously high values were obtained but the error was about one-tenth of that incurred with the method of Fiske & Subbarow. With the precipitation method, on the other hand, the recoveries of inorganic phosphate were low.

The blue pigment which developed in absence of inorganic phosphate could be extracted from acid solutions by *iso*butanol, contrary to statements contained in the literature. It was partly extracted when the acidity was 0·5 N, and completely, when the acidity was 1 N or higher.

DISCUSSION

Our results suggest that the effect of Lutwak & Sacks is only observed if a blue colour develops during the period of heating with molybdate. The colour development may be attributed to the reducing action of the organic component of the phosphate ester. No colour development occurred in the experiments reported in our first paper (Weil-Malherbe & Green, 1952) and accordingly no indication of the effect was obtained. On several occasions hydrolysis was allowed to go to completion, but colour intensities exceeding the theoretical value were not observed (see, for example, Fig. 13, Weil-Malherbe & Green, 1952). In the present work no colour developed at pH 5·5 and 80° and the phosphate estimations by the three methods were in agreement. Contrary to the claims of Lutwak & Sacks their figures do not bear out the existence of the effect in those of their experiments carried out at pH 5·5 and 100°. If the hydrolysis estimated by the precipitation method is expressed as a percentage of the hydrolysis estimated by the direct colorimetric method, the mean 'recovery', calculated from their table 1, is 70·6% (S.E. 16·4%) after heating without molybdate (columns 3 and 6), and 99·5% (range 88–113%) after heating at pH 5·5 with molybdate (columns 2 and 5). The agreement is thus in fact better in the experiments with molybdate. Even in the case of phenyl phosphate, specifically quoted by Lutwak & Sacks, the results agree within 12%, which is well within the standard deviation of the series without molybdate where 'good agreement was obtained between the values found by the two procedures'.

The explanation which attributes the effect to the formation of organophosphomolybdate complexes does not seem plausible. It is improbable, though not impossible, that a complex should be formed from molybdate, fructose and inorganic phosphate which would behave similarly to a complex of molybdate with fructose 6-phosphate. Far from increasing colour development, organic phosphates, which form complexes with molybdate (e.g. α-

Table 3. *Specific extinction coefficients in various methods of phosphate estimation*

Method	Temperature of colour development	Reducing agent	Wavelength (mμ.)	$E^{1\,g.\,P/l.}_{1\,cm.}$	Ref.
Fiske & Subbarow	About 20°	ANS*	730	126	This paper
Fiske & Subbarow	About 20°	ANS	Approx. 700	150	Norberg (1942)
Teorell	About 20°	ANS	Approx. 700	195	Teorell (1931)
Griswold *et al.*	100°	ANS	820	860	Griswold, Humoller & McIntyre (1951)
Lowry & Lopez	About 20°	Ascorbic acid	700	140–150	Lowry & Lopez (1946)
Sumner	About 20°	$FeSO_4$	730	114	This paper
Boltz & Mellon	100°	Hydrazine	830	864	Boltz & Mellon (1947)
Denigès	About 20°	$SnCl_2$	Approx. 700	700	Norberg (1942)
Berenblum & Chain	About 20°	$SnCl_2$	Approx. 700	700	Norberg (1942)
Martin & Doty	About 20°	$SnCl_2$	730	740	This paper
Fontaine	100°	$SnCl_2$	820	950	Fontaine (1942)

* ANS = 1-Amino-2-naphthol-4-sulphonic acid.

glycerophosphate), inhibit it (Davies & Davies, 1932), at any rate at room temperature. In the absence of further evidence other explanations ought to be considered. In our view the effect is caused by three factors which, together, fully account for it.

The first factor is a decreased recovery of inorganic phosphate by precipitation with magnesia mixture after the reduction of the phosphomolybdate complex. This may be due to a greater resistance of the reduced complex to alkali.

The second factor is the formation of molybdenum blue, due to the reduction of free molybdic acid. Contrary to prevailing opinions, molybdenum blue is extracted from acid solutions by *iso*butanol. It is likely that this factor was more prevalent in the experiment in which free fructose and inorganic phosphate were heated with molybdate than in the experiment with fructose 6-phosphate. But its occurrence in the latter cannot be entirely ruled out.

The third factor is an increase of the specific extinction coefficient of the reduced phosphomolybdate complex with temperature. Table 3 contains some values of specific extinction coefficients for phosphorus, obtained by various methods of phosphate estimation. Some were collected from the literature, others were determined in this laboratory with the Unicam spectrophotometer. It appears that the effect of temperature on the specific extinction coefficient is much greater for the reducing agent of Fiske & Subbarow (aminonaphtholsulphonic acid) than for stannous chloride. If the colour is developed at 100°, all reducing agents for which data are available lead to an extinction of about 800–900. Stannous chloride is the only reducing agent so far known with which a similar extinction is already obtained at room temperature. As has been shown by Berenblum & Chain (1938), only two molybdate groups are reduced per atom of phosphorus in the method of Fiske & Subbarow, whereas nine are reduced in the method of Kuttner & Cohen (1927) employing stannous chloride. A slow intensification of colour takes place in the method of Fiske & Subbarow which continues for more than 72 hr. (McCune & Weech, 1940), while the colour produced by stannous chloride is stable. It may be suggested that the reduction of the first two molybdate residues of the phosphomolybdate complex is much faster than the reduction of the next seven and that stannous chloride alone is a sufficiently strong reducing agent to bring about the reduction of all nine groups in a short time at room temperature. At a temperature of 100° the reduction of all nine groups is fast even with the weaker reducing agents.

It is probable, therefore, that the effect of Lutwak & Sacks is largely due to the fact that the colour was developed under conditions which produce an extinction about 4–5 times higher than that which is produced in the reaction used for the preparation of the reference solution. Stannous chloride methods are much less subject to, even perhaps quite free from, this error, and are therefore more suitable for the investigation of the problem under discussion.

SUMMARY

1. Lutwak & Sacks (1953) recently found that, when solutions of organic phosphates were heated with molybdate, estimations of inorganic phosphate by direct colorimetry and by colorimetry after previous precipitation did not agree.

2. It has now been shown that this effect is observed only when a blue colour develops during the exposure to heat. The effect is similar whether the solution contains, e.g. fructose phosphate or a mixture of fructose and phosphate, and is therefore unlikely to be due to the formation of organophosphomolybdate complexes.

3. It is suggested that the effect is due to three factors. (*a*) If magnesia mixture is added to the reduced phosphomolybdate complex and the resulting precipitate analysed colorimetrically, recoveries of inorganic phosphate are low. (*b*) Free molybdic acid is reduced, giving molybdenum blue. Contrary to prevailing opinions, molybdenum blue is extracted from acid solutions by *iso*butanol. (*c*) The specific extinction coefficient is about 4–5 times higher when the colour of the reduced phosphomolybdate complex is developed at 100° than when it is developed at room temperature. This is true for most reducing agents except stannous chloride, with which a higher extinction is obtained at room temperature.

4. The effect is not observed under the milder experimental conditions of Weil-Malherbe & Green (1952). If it had occurred, a stannous chloride method of phosphate estimation, such as was used by these authors, would have been less subject to error than a method using aminonaphtholsulphonic acid.

The author is indebted to Mr R. H. Green for much technical assistance.

REFERENCES

Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 286.
Boltz, D. F. & Mellon, M. G. (1947). *Ind. Engng Chem. (Anal.)*, **19**, 873.
Davies, D. R. & Davies, W. C. (1932). *Biochem. J.* **26**, 2046.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Fontaine, T. D. (1942). *Ind. Engng Chem. (Anal.)*, **14**, 77.
Griswold, B. L., Humoller, F. L. & McIntyre, A. R. (1951). *Analyt. Chem.* **23**, 192.
Kuttner, T. & Cohen, H. R. (1927). *J. biol. Chem.* **75**, 517.
Lowry, O. H. & Lopez, J. A. (1946). *J. biol. Chem.* **162**, 421.
Lutwak, L. & Sacks, J. (1953). *J. biol. Chem.* **200**, 565.
McCune, D. J. & Weech, A. A. (1940). *Proc. Soc. exp. Biol., N.Y.*, **45**, 559.
Martin, J. B. & Doty, D. M. (1949). *Analyt. Chem.* **21**, 965.
Norberg, B. (1942). *Acta physiol. scand.* **5**, suppl. 14.
Teorell, T. (1931). *Biochem. Z.* **230**, 1.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques and Related Methods for the Study of Tissue Metabolism.* Minneapolis: Burgess Publishing Co.
Weil-Malherbe, H. & Green, R. H. (1952). *Biochem. J.* **49**, 286.

Studies with Marked Antisera

QUANTITATIVE STUDIES WITH ANTISERA MARKED WITH IODINE ^{131}ISOTOPE AND THEIR CORRESPONDING RED-CELL ANTIGENS

By J. C. BOURSNELL, R. R. A. COOMBS and VIVIEN RIZK
Departments of Biochemistry, Animal Pathology and Pathology, University of Cambridge

(*Received* 8 *August* 1952)

By now a considerable number of workers has begun to use the radioisotope technique in investigations on serological and immunological phenomena (see review by Francis, Mulligan & Wormall, 1951). These methods enable one to trace quantitatively suitably marked antibodies and antigens in amounts beyond the range of any other known methods. The object of the present investigations was the study of the combination of specific serum antibodies with antigens on the red-cell surface by quantitative isotopic methods. Such a study entails marking the antibody molecules in an antiserum with an isotope and allowing the marked antiserum to react with a suspension of red blood cells. The cells are then washed free of uncombined protein and the radioactivity of the washed, sensitized cell suspension measured.

Because of the necessity of washing the sensitized cell suspensions free of all uncombined serum proteins, antigen-antibody systems had to be chosen which do not produce strong agglutination or clumping of the cells, since it would not be possible to wash the aggregates free of all protein other than that specifically bound to the cell, and haemolysis would probably occur if an attempt were made to break them up by mechanical agitation. The following red-cell antigen-antibody systems are suitable. (1) The so-called incomplete Rh antibodies (Race, 1944; Wiener, 1944) combine with the red-cell antigens but for some reason, as yet unknown, fail to bring about agglutination. We have used the incomplete anti-D Rh antiserum with Rh-positive human red cells. (2) The red cells of certain oxen, owing either to the disposition of the antigens on the cell surface, or to some other physical property of their surface, remain unagglutinated when sensitized by potentially agglutinating antibodies. We have used bovine red cells of the 'in-

agglutinable' type (Gleeson-White, Heard, Mynors & Coombs, 1950) with either rabbit anti-bovine red-cell serum or human serum containing the Paul-Bunnell antibody which also has a specific affinity for an antigen on bovine red cells.

The isotope used to mark the antibodies in the sera was elementary radioactive iodine (^{131}I; half-life 8·0 days). The use of iodine as a tracer has many distinct advantages. The iodine isotope is easy to measure, has a conveniently long half-life for *in vitro* studies, and is readily obtainable. If radioactive iodine, in the presence of a suitable quantity of 'carrier' iodine, is allowed to react with protein at physiological pH and at room temperature, in such a way that about one atom is introduced per protein molecule, the iodine appears to be very firmly bound. Moreover, the serological titre of the antibody is not apparently affected by this treatment if allowance is made for the dilution of the antiserum which occurs during iodination.

Early in the experimental work with these serological systems a serious obstacle was encountered in the large adsorption by red cells of marked serum proteins which could not be detected serologically and which appeared to be entirely non-specific. However, once this difficulty was overcome and assurance was gained that the methods were reliable, the investigations were continued along the two following lines: (*a*) measurement of the total specific antibody content of an antiserum in terms of μg. protein; (*b*) determination of the total number of sites of a particular antigen on a red-cell surface.

The quantitative results offer an explanation for certain empirical findings associated with the serological systems studied.

METHODS AND MATERIALS

Preparation and estimation of radioactive materials

Iodination of proteins. A study was made of the best methods available to liberate iodine quantitatively from the iodide, the form in which ^{131}I is normally transmitted from the Atomic Energy Research Establishment. It was felt that, if possible, the resulting iodine solution should contain nothing that could be considered deleterious to the protein. For this reason attention was concentrated on the reaction $KIO_3 + 5KI + 6HCl = 3I_2 + 3H_2O + 6KCl$ (cf. Francis *et al.* 1951). As normally used, in this reaction both the KI and the HCl are present in considerable excess, and the reaction proceeds to completion. However, it is desirable to avoid a large excess of HCl in the subsequent reaction with the antiserum and the need to conserve radioactive iodine means that the KI must not be present in large excess either. It was found that iodine was still liberated quantitatively from the KI with a slight excess of KIO_3 and HCl according to the above equation. Further investigation of this reaction in which an attempt was made by the addition of a buffer to the system after the liberation of the iodine, to reduce the pH to more nearly physiological conditions, revealed the fact that apparently the reaction does not give a quantitative liberation of iodine under these conditions. However, the loss entailed was not serious, and as a standard procedure the solutions were added in the following order to a stoppered test tube:

(*a*) 0·11 ml. KIO_3 (0·107 g./100 ml.).

(*b*) 0·1 ml. KI (0·415 g./100 ml.) to which had previously been added the $Na^{131}I$ solution (usually in small volume).

(*c*) 4 drops 0·1 N-HCl

(*d*) 2 drops 0·2 M phosphate buffer (pH 8·0).

(*e*) The serum to be iodinated (usually between 0·5 and 2·0 ml.).

The serum was always added as quickly as possible after the buffer and the reaction was allowed to proceed for 45 to 90 min. Any trace of iodine remaining was removed by the addition of a few drops of 0·1 N-$Na_2S_2O_4$.

The resulting solution was dialysed in cellophan tubing against 500 ml. quantities of saline (0·9% (w/v) NaCl) in the cold (1–4°) until the dialysing saline was free from radioactivity. Five changes of saline at intervals of 5 hr. or more sufficed.

It follows that in the iodination procedure and dialysis a certain dilution of the serum protein is incurred. The concentration of the ^{131}iodinated protein was measured by means of Kjeldahl total-N determinations; in serial dilutions, this is designated 'neat'.

Observations upon the stability of the iodine-protein linkage. An attempt was made to determine the stability of the iodine attached to the serum proteins used in these experiments. A quantity of ^{131}I-marked rabbit anti-bovine red-cell serum, after dialysis as described above, was placed at the bottom of a U-shaped fold of new cellophan tubing to avoid any leakage through the tied ends of the sac. The contents of the sac were dialysed against 10 ml. quantities of saline both at 4° and at room temperature for a fortnight. During this time only a very small proportion (2·6%) of the total radioactivity (allowing for decay) escaped into the saline dialysing medium. It seemed likely that this could be accounted for by a slight reduction in the total quantity of protein present in the sac, due either to a very slow leakage of protein or possibly to bacterial action.

It was found that there was no fixation by red blood cells of quantities of iodide or iodine very much greater than was ever likely to be liberated by the rupturing of the iodine-protein linkage.

The relative combination of iodine with serum and γ-globulin. A study was made of the ^{131}I/total-N ratios in marked whole human serum and in γ-globulin separated therefrom. Preliminary experiments had shown that the ratio for the separated γ-globulin was somewhat less than that for the whole serum (cf. Pressman & Sternberger, 1950). By iodinating with different quantities of added carrier iodine, following in general the method described above, samples of marked serum were obtained with iodine contents varying from about 0·07 to 2 atoms/molecule of protein (assumed average mol.wt. of 1×10^5).

The γ-globulin was separated by using 18 and 12% (w/v) Na_2SO_4 according to the method of Kekwick (1940). Samples of the initial ^{131}I-marked sera and of the γ-globulin were taken for radioactivity and total-N determinations. From these figures the specific activity in terms of counts/min./μg. protein could be calculated. In order to make possible the calculation of the atoms of iodine/molecule of protein, it was necessary to know the specific activity (counts/min./μg. iodine) of the liberated iodine in each case. This was facilitated by making up the original ^{131}I sample

to a known volume. Portions of this were used for each iodination, and from this also radioactivity standards were prepared. From these figures, accepting an average mol.wt. of 1×10^5 for serum proteins, the atoms of iodine/protein molecule can readily be calculated.

The graph (Fig. 1) shows that the ratio (I/total N in γ-globulin to I)/(total N in whole serum proteins) varies with the average number of atoms of iodine on the serum protein molecules, and passes through a maximum at about one atom of iodine/molecule.

A subsidiary experiment in which separated γ-globulin was iodinated and then reprecipitated with 18% Na_2SO_4 showed that this treatment did not decrease the iodine content of the protein. Moreover, the warming of the precipitating mixture to 35°, which is necessary with Na_2SO_4 precipitation, does not appear to affect the iodine-protein link. This is important because in all the experiments incubation for sensitization was carried out at 37°.

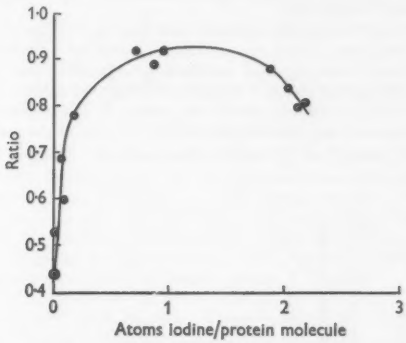


Fig. 1. Curve showing relationship between the average number of atoms of iodine per protein molecule (assumed mol.wt. of serum proteins, 1×10^5) and the ratio expressed as I/total N in γ-globulin to I/total N in whole serum proteins.

Pressman & Sternberger (1950), using precipitates of egg albumin and ^{131}I-marked rabbit anti-egg albumin serum, have shown that the iodine uptake by the antibody was less than that of the total globulin fraction of the serum. There appears to be a discrepancy between the observations in the literature relating to the relative rate of iodination of various serum components. The above authors found that with six or more iodine atoms introduced, on the average, into rabbit antisera, the rate of iodination of the specifically precipitable antibody was less than that of the globulin which was again less than that of whole serum. The presence of antibody was still detectable under these conditions. Masouredis, Melcher & Koblick (1951), on the other hand, using 1·3 iodine atoms in globulin prepared from rabbit antisera without any apparent destruction of antibody, find that the specifically precipitable antibody has an identical iodine content, assuming a mol.wt. of $1{\cdot}6 \times 10^5$ for both. Cohen (1951) has found that iodination of equine antitoxic serum gives a globulin whose rate of iodination is 90–94% of that of the average serum proteins. Pressman & Sternberger (1950) also show that in iodination of mixed rabbit antisera to ovalbumin and to bovine serum albumin the relative rates of iodination of the specifically precipitable antibodies are identical.

Unfortunately, the methods that these authors used to determine the relative rate of iodination of antibody cannot be applied to cellular antigen systems, and therefore, until evidence is available on the point in the particular systems studied here, the γ-globulin-I:serum-I ratio has been taken to apply (in series E) to the antibody fraction also. It is realized that the application of this ratio may not give absolute values.

Radioactivity determinations

Mounting the samples. Radioactivity standards were made as follows: one small drop of the marked protein solution was weighed out and diluted (by weighing) 20–30 times with a 1/10 solution in saline of normal bovine serum. It was found that, by using this 1/10 serum as a diluent, much better reproducibility of the standards was obtained than when normal saline was used alone. After thorough mixing, drops of the diluted ^{131}iodinated protein were weighed out on to concave nickel planchets (General Electric Company, Magnet House, Kingsway, London, W.C. 2) which had previously been thinly lacquered with a pale-gold lacquer. One drop of a 3% solution of polyvinyl alcohol in water was then added and the planchet allowed to dry on a warm hot-plate. The sandwiching of the radioactive material between the lacquer and the polyvinyl alcohol, both of which appeared to be necessary, completely eliminated the tendency of the dried material to flake or curl. It was also found that, by this treatment, the absorption of the β- and γ-radiation from the ^{131}I was only slight, and was in any case sensibly constant for all the samples and standards.

Counting methods and expression of results. The radioactivities of the dried preparations on the planchets were determined by means of conventional, end-window, commercial Geiger-Müller counters and electronic scalers. Two and sometimes three of these sets were used in the radioactivity assays in any one experiment.

The standard samples, five in number for each serum, were usually counted at a definite time in the course of the day, and from these an average figure for the counts/min./μg. ^{131}iodinated protein was obtained. The ratio (representing μg. ^{131}iodinated serum protein on the planchet) of the cell sample count to this average figure was obtained at least twice and on different counting sets, if possible on different days. In a considerable proportion of the cases two determinations of the ratio sufficed. Occasionally more than three determinations were required and this could often be explained by the unreliability of the counts during the first hour after switching on the counting sets. Usually the maximum activity of any of the planchets was between 100 and 500 counts/min., and the minimum activity was 10 or less. It is difficult to work out the overall error arising from such variables as cell sampling and small unavoidable losses in the transference of cells, but the counting errors are certainly less than 5% in almost all cases.

Determination of the error in counting due to self-absorption. Experimental determination of the magnitude of this effect showed that, with about 360×10^6 cells (equivalent to 0·5 ml. 4% cells) on a planchet, the self-absorption was less than 3%. With larger quantities of cells the self-absorption became rapidly appreciable, and to avoid the application of a correction factor, in these cases the cell suspension was divided and mounted on two planchets.

Serological materials and methods

Red cells. Bovine and human red blood cells have been used in these experiments. Defibrinated blood was obtained twice weekly from cow 5157. The cells of this animal belonged to the 'inagglutinable' class described by Gleeson-White *et al.* (1950). Human blood (Rh-positive and Rh-negative) was taken by venepuncture into Wintrobe's oxalate mixture (Wintrobe, 1942). Some samples were taken by pricking the ear and collecting the blood in 0·6% citrate-saline (6 g. trisodium citrate in 1 l. saline). The only Rh antigen we have employed has been the D antigen, and for this work red cells are referred to as Rh-positive if they possess this antigen, Rh-negative if they do not. The probable antigenic structure of the cells is indicated in each case (e.g. (CDe/cde), cf. Race & Sanger, 1950).

The cells were washed three times in saline before use, and the final suspension of known concentration was made in saline or other diluent as indicated in the individual experiments. The final cell concentrations were checked by counting the cells in a haemocytometer.

Sera. (*a*) *Rabbit anti-bovine red-cell sera.* Serum was obtained from rabbit 1876 before and after one and two courses of injections with washed bovine red cells. Each course consisted of six intravenous injections of 1 ml. 10% cell suspension.

The haemolysin titres were estimated by titrating the heat-inactivated sera in 0·1 ml. amounts and adding 0·1 ml. 1% bovine red-cell suspension and 0·1 ml. (4 minimum haemolytic doses) of guinea pig complement. The degree of haemolysis was read after 30 min. incubation at 37°. The titre of the pre-injection serum was less than 5, while after the first course of injections it was 1280, and 5120 after the second course.

(*b*) *Human anti-bovine red-cell sera.* Human serum containing the Paul-Bunnell antibody reactive against bovine red cells was obtained from a patient (*C*) who had contracted infectious mononucleosis. The haemolysin titre of this serum for bovine red cells was 5120. Normal human sera have been used in control tests.

The sera in (*a*) and (*b*) were heat-treated at 56° for 30 min. before iodination. Certain samples, as indicated in the individual experiments, were absorbed with bovine red cells also before iodination.

(*c*) *Human incomplete anti-D Rh sera.* The sensitization of Rh-positive red cells by non-agglutinating incomplete antibodies in Rh antisera may be demonstrated by the anti-globulin sensitization test (Coombs, Mourant & Race, 1945). In this test the cells, after sensitization, are washed free of unabsorbed protein and then tested for adsorbed antibody globulin by means of a rabbit anti-human globulin serum. Sensitized cells are agglutinated by such an anti-globulin serum.

By this procedure the titre of incomplete antibody (anti-D) was 1024 in serum *W* and 2048 in sera *B* and *Bgl.* (Serum *B* contained a trace of incomplete anti-C (titre 8) also.) These human sera were not heat-inactivated before use, but the testing systems were compatible with regard to the ABO blood grouping systems.

The reactions of anti-Rh sera may be controlled serologically either by using Rh-negative cells or by using normal sera on Rh-positive cells. Neither of these two procedures, however, is without certain limitations as a control for the non-specific adsorption of marked protein. The main limitation is inherent in the fact that the non-specific adsorption appears to vary slightly from serum to serum and also with cell samples from different individuals.

The effect of iodination on the antibody titre. Four samples of heat-inactivated infectious mononucleosis serum (*C*) were iodinated, each with increasing amounts of carrier iodine as described previously. After dialysis all the ^{131}I-marked sera were adjusted to the same protein concentration as was also an un-iodinated dialysed control sample. Each sample was titrated for its haemolysin end point against bovine red cells. Tests were made with both doubling and four-fifth progressive dilutions of the samples.

The samples 1–4 contained 0·03, 0·2, 1·8 and 2·7 iodine atoms/protein molecule respectively. No drop in the serological titre could be detected when compared with the control except in sample 4. Even in this case, the last tube of the 'four-fifth' titration to show any haemolysis was the same as in the control, but the haemolysis was definitely weaker in this tube.

From these results it would seem that an average of 1·8 iodine atoms/serum protein molecule can be introduced without destroying any antibody or interfering with its combining properties. A similar experiment carried out with the incomplete anti-D Rh serum *B* again gave no evidence of any effect on the serological titre of the serum with as much as 1·8 iodine atoms/molecule. These results

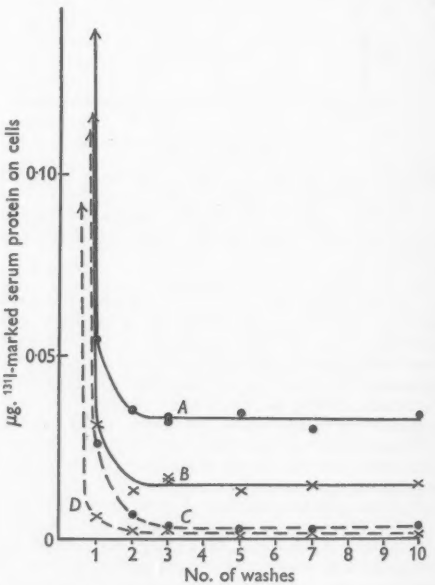


Fig. 2. The effectiveness of washing Rh-positive and -negative human red cells with saline after previous treatment with 0·1 ml. ^{131}I-marked incomplete anti-D serum (^{131}I-*B*, protein 0·11%). Curve *A*, 0·5 ml. 4% suspension (A CDe/CDe) cells; curve *B*, 0·5 ml. 1% (A CDe/CDe) cells; curve *C*, 0·5 ml. 4% (A cde/cde) cells; curve *D*, 0·5 ml. 1% (A cde/cde) cells.

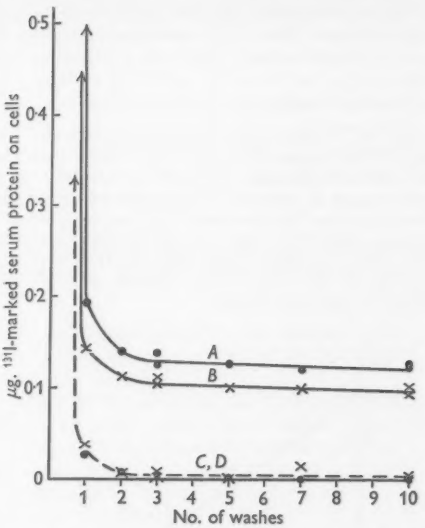


Fig. 3. The effectiveness of washing 'inagglutinable' bovine red cells (0·5 ml. 1% suspension) with saline after previous treatment with 0·1 ml. serum at 37°. ^{131}I-marked human infectious mononucleosis serum (protein 0·26%), incubated for curve *A*, 40 min.; curve *B*, 5 min. ^{131}I-marked normal human serum (protein 0·24%), incubated for curve *C*, 40 min.; curve *D*, 5 min.

are in general agreement with the observations of Masouredis *et al.* (1951) with rabbit antibody and of Cohen (1951) using equine diphtheria antitoxin.

Measurement of the adsorption of ^{131}I-marked antibody on red cells. The following procedures were used in all the experiments, unless otherwise stated.

The marked serum was 'titrated'* with 'doubling dilutions' in a constant volume. To this 'titration' was added equal volumes of a known concentration of red cells. In the early experiments saline was used as the diluent for the serum and red-cell suspensions, but in later experiments this was replaced by undiluted heat-inactivated unmarked serum compatible with the test. In these later tests the red-cell suspensions were prepared 1 hr. before they were added to the 'titrations'. The contents of the tubes were mixed and incubated at 37° for 40 min. The tubes were then centrifuged and the packed cells washed three times with 2 ml. quantities of saline. The final deposit of cells in each tube was resuspended in three drops of saline and transferred to a planchet for radioactivity determinations.

Investigation of the effectiveness of washing treated cells with saline. In these experiments an attempt was made to investigate the relative effectiveness of repeated washing with saline of cells treated with ^{131}I-marked sera.

(*a*) *Using ^{131}I-marked anti-D Rh serum* (^{131}I-B). Eight tubes were set up each containing 0·1 ml. ^{131}I-*B* and 0·5 ml. 1% suspension of Rh-positive cells in unmarked heat-inactivated human serum. A similar series of tubes were set up with Rh-negative cells. The contents of the tubes were incubated, centrifuged and the resulting eight cell deposits of each series were washed 0, 1, 2, 3, 3, 5, 7 and 10 times respectively. Each washing was with 1·6 ml. saline. The whole experiment was repeated using a 4% cell suspension with comparable results as shown in Fig. 2. It will be seen that by three washes all the loosely attached marked protein had been removed and no appreciable further removal took place even with ten washes.

(*b*) *Using ^{131}I-marked infectious mononucleosis serum* (^{131}I-C). A similar experiment was performed with bovine red cells treated with ^{131}I-*C* and with ^{131}I-marked normal human serum. In this case, only one concentration of cells (1%) was used but two different periods for sensitization were employed (5 min. and 40 min.). The findings (Fig. 3) were identical to those of (*a*).

In the experiments described in series D, it was necessary to transfer as quantitatively as possible all the free ^{131}I-marked serum with the least possible increase in bulk. In the two experiments, (*a*) and (*b*) above, determinations show that only 1–2% of the total quantity remained trapped in the cell deposit before washing. In (*a*), washing this deposit with one drop of saline removed 60% or more of this remaining ^{131}I-marked serum. For this reason initial washing with one drop of saline was employed in the experiments in series D.

EXPERIMENTAL

Series A. 'Titrations' of marked antisera against red-cell suspensions in saline

Expt. A 1. *'Titration' against bovine red cells of ^{131}iodinated γ-globulin prepared from rabbit anti-bovine red-cell serum.* The γ-globulins for this experiment were prepared from pre-injection and second course sera from rabbit 1876 by three precipitations at 1·39M concentration of $(NH_4)_2SO_4$, following closely the method of Cohn, McMeekin, Oncley, Newell & Hughes (1940).

The γ-globulins were iodinated separately as described above, and 'titrated' in 0·1 ml. amounts with a 1% suspension of bovine red cell. The haemolysin titre of the γ-globulin preparation of the antiserum, both before and after iodination, measured under comparable conditions, was 512.

The results, together with other relevant data, are shown in Fig. 4. It is of interest to note that radioactivity is still detectable on the cells beyond the serological end point. The slight adsorption from the pre-injection ^{131}iodinated γ-globulin might have been due to a small amount of naturally occurring antibody, but was probably non-specific.

Expt. A 2. *'Titration' of ^{131}I-marked rabbit anti-bovine red-cell serum (1st course) and ^{131}I-marked infectious mononucleosis serum against bovine red cells.* The two antisera and two appropriate normal sera were heat-inactivated, and the two normal sera were then absorbed free of antibodies for bovine red cells. After iodination, the ^{131}I-marked sera were 'titrated' in 0·2 ml. amounts with an equal volume of a 1% suspension of bovine red cells.

* In this work 'titration' is used to indicate that the procedure carried out follows that of a normal serological titration (but without the possibility of an end point).

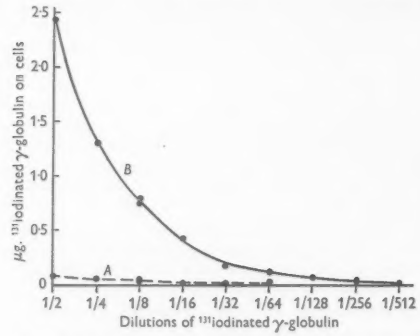


Fig. 4. 'Titration' (curve *A*) of ^{131}I-marked rabbit γ-globulin ('neat', 0·22% protein) from pre-injection serum; (curve *B*) ^{131}I-marked rabbit γ-globulin ('neat', 0·27% protein) from second course anti-bovine red-cell serum against 0·1 ml. 1% suspension of bovine red cells in saline.

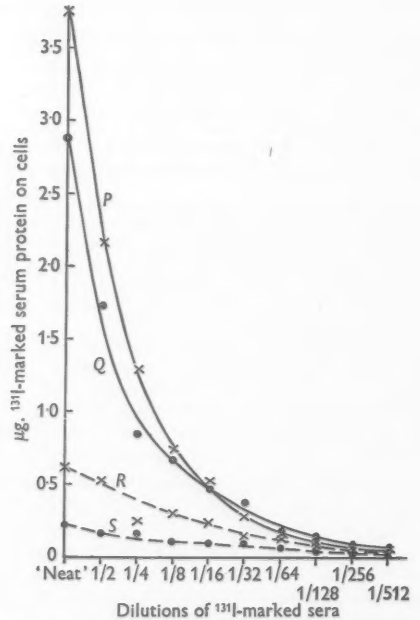


Fig. 5. Comparative 'titrations' of ^{131}I-marked rabbit anti-bovine red-cell serum and ^{131}I-marked human infectious mononucleosis serum (^{131}I-*C*) against 0·2 ml. 1% suspension of bovine red cells in saline. Curve *P*, ^{131}I-*C* ('neat', 2·6% protein); curve *Q*, ^{131}I-marked rabbit anti-bovine red-cell serum ('neat', 2·3% protein); curve *R*, ^{131}I-marked normal human serum ('neat', 2·6% protein); curve *S*, ^{131}I-marked preinjection rabbit serum ('neat,' 2·0% protein).

Fig. 5 shows that the two antisera gave comparable results. The quite considerable amount of marked protein adsorbed from the two normal sera could not be due to specific antibody as these sera had been previously absorbed with bovine red cells.

Expt. A 3. *'Titration' of* ^{131}I*-marked incomplete anti-D Rh serum* ^{131}I-W) *against Rh-positive and -negative human red cells.* In this experiment only one serum was used, ^{131}I-*W*, which showed no reduction of its sensitizing titre of 1024 after iodination. The specificity of the serological reaction was controlled by 'titrating' the serum against Rh-negative cells, as well as against Rh-positive cells. The 'titrations' were carried out using 0·1 ml. amounts of ^{131}I-*W* and 0·1 ml. 1% cell suspensions.

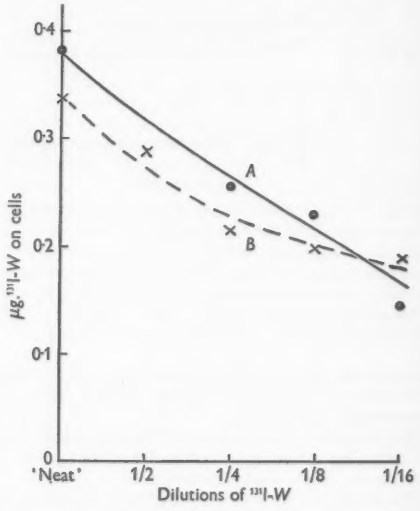


Fig. 6. 'Titrations' of 0·1 ml. ^{131}I-marked incomplete anti-D human serum (^{131}I-*W*, 'neat', 2·3% protein) against 0·1 ml. 1% saline suspensions of Rh-positive and -negative cells. Curve *A*, Rh-positive (O cDE/cde) cells; curve *B*, Rh-negative (O cde/cde) cells.

From Fig. 6 it may be seen that there could be no certainty of differentiating at any dilution of the serum between the Rh-positive and -negative cells by the adsorption of ^{131}iodinated protein. Yet serologically the differentiation was unequivocal, even at a dilution of 1/1024. The explanation of these results, as was made evident by subsequent experiments, is that the specific adsorption on the Rh-positive cells is very small in comparison with the non-specific adsorption.

Observations on the experiments in series A. In the experiments with antigens on bovine red cells the specific adsorption of the ^{131}I-marked antibody was clearly differentiated from the non-specific. In contrast the results with human cells and the in-

complete anti-D serum suggested that the specific antibody adsorption was very small and was in fact practically overshadowed by the non-specific adsorption.

It was obvious from these results that this quantitative study of specific antibody adsorption could not progress until the problem of non-specific adsorption had been overcome. The experiments in series B were devoted to this problem.

Series B. Reduction of the apparently non-specific adsorption of ^{131}I-marked serum proteins on to red cells

A number of attempts have been made to reduce the non-specific adsorption of ^{131}I-marked serum proteins on to red cells.

(*a*) *Effect of temperature.* As this non-specific adsorption by a saline suspension of cells brought to mind serological phenomena described under such terms as 'non-specific cold agglutination' and 'cold autoagglutination' it was decided to study the influence of temperature on the adsorption. A serologically compatible system was chosen for study consisting of ^{131}-iodinated γ-globulin from rabbit serum and a saline suspension of cells from the same animal, and the interaction studied at 16 and 39°. Although the non-specific adsorption was reduced at the higher temperature, it was by no means completely abolished. Adsorption to about the same extent also took place from a ^{131}I-marked rabbit albumin solution, a finding which supported the probability that the adsorbed protein was not specific antibody.

(*b*) *Effect of citrate.* Dacie (1950), in describing an apparently non-specific adsorption of globulin from unheated human serum on to human red cells in the cold, found that the adsorption could be partially inhibited by 0·6% sodium citrate. However, the use of this medium did not prevent the adsorption under discussion here.

(*c*) *Prior adsorption of unmarked protein.* The red cells, after being washed in saline and before use in the experiment, were suspended for 1 hr. at room temperature in antibody-free heat-inactivated unmarked serum, to enable the cells to adsorb non-specifically unmarked serum proteins before coming into contact with the ^{131}I-marked serum. In addition serial dilutions of the ^{131}I-marked sera were carried out with the same unmarked serum instead of with saline. It was hoped that this treatment would decrease the non-specific adsorption of marked protein while still allowing the specific antibody adsorption to proceed unimpaired. The two following experiments demonstrate the use of unmarked serum as diluent for cells and serum to reduce the non-specific adsorption.

Expt. B1. Bovine red cells with ^{131}I-marked infectious mononucleosis serum and ^{131}I-marked normal human serum. The details of this experiment are shown in Fig. 7. The results show that it is possible to reduce the non-specific adsorption to a very low value by using unmarked serologically compatible serum as the diluent. The effect is the same whether bovine or human serum is used. Washing the cells with serum rather than saline did not reduce the non-specific adsorption any further. The reductions due to this treatment were similar in the two sera and the resulting clearly defined difference between the two adsorptions presumably represented specific antibody. Thus the non-specific adsorption had been largely removed while leaving the specific antibody adsorption apparently unchanged.

Expt. B2. Rh-positive and -negative cells with ^{131}I-marked incomplete anti-D Rh serum (^{131}I-B). The cells used in this experiment were Rh-positive and Rh-negative. 1% suspensions were made in saline and also in heat-inactivated normal human serum from an O Rh-negative person. 'Titrations' in 0·1 ml. amounts of ^{131}I-*B* were set up as indicated in Fig. 8.

After the usual incubation, the cells were centrifuged and washed in saline since, as found in expt. B1, washing with unmarked serum does not further reduce the non-specific adsorption. The results of these 'titrations' are shown in

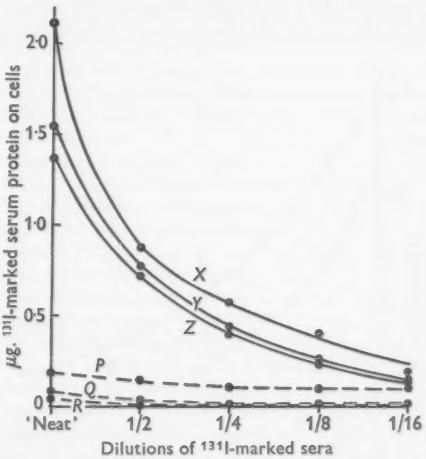


Fig. 7. The use of unmarked serum as diluent for cells and serum to reduce the non-specific adsorption of ^{131}iodinated protein on to bovine red cells. 'Titrations' of 0·1 ml. ^{131}I-marked normal human serum (absorbed with bovine red cells, 'neat', 2·5% protein) against 0·1 ml. 1% bovine cells: diluent for cells and ^{131}I-marked serum, curve *P*, saline; curve *Q*, human serum; curve *R*, bovine serum. Similar 'titrations' of ^{131}I-marked infectious mononucleosis serum ('neat', 3·0% protein): diluent for cells and ^{131}I-marked serum, curve *X*, saline; curve *Y*, human serum; curve *Z*, bovine serum. The cell suspensions were made from a 10% saline suspension by diluting with either (*a*) saline, (*b*) heat-inactivated human AB serum (absorbed with bovine red cells) or (*c*) heat-inactivated bovine serum.

Fig. 8. By the previous incubation of the cells in unmarked serum, the adsorption of ^{131}iodinated protein on to both the positive and negative cells was reduced very considerably, although the difference between the adsorption on positive and negative cells remained unchanged.

Observations on the experiments in series B. The use of unmarked compatible serum as a diluent for both cells and ^{131}I-marked sera apparently did not interfere with antibody adsorption in any way that could be shown serologically or by isotope measurements. At the same time this procedure overcame sufficiently the complication due to the non-specific adsorption to allow quantitative studies on the adsorption of ^{131}I-marked antibody to be pursued. From these and other experiments not reported here it can be stated that the higher the ratio (in terms of total-N concentration) of unmarked serum to ^{131}I-marked anti-D Rh serum the more effective it is in reducing the non-specific adsorption. This treatment is not satisfactory when the ratio is less than 20.

The amount of specific anti-D antibody that was adsorbed on to Rh positive cells appeared to be very small when compared with the amount of ^{131}I-marked antibody that was adsorbed on to bovine red cells from either infectious mononucleosis serum or a rabbit anti-bovine red-cell serum. The smallness of this adsorption seemed puzzling at first as the anti-D serum used had a very high antibody titre, but the results could be interpreted as suggesting that there are relatively few D-receptors on Rh-positive cells. Experiments in the following series support this explanation.

Series C. The effect of the red-cell concentration on antibody adsorption

*Expt. C*1. *With ^{131}I-marked infectious mononucleosis serum* (^{131}I-C). Bovine red-cell suspensions were made in heat-inactivated unmarked human serum which had previously been absorbed with bovine cells. This same serum was also used as a diluent for the ^{131}I-marked serum 'titrations'.

Suspensions of 4, 3, 2, 1 and 0·5% cells were made up and 0·5 ml. quantities of each respectively were added to a series of comparable 'titrations' of ^{131}I-*C* in 0·1 ml. amounts. 'Titrations' using the 4 and 0·5% cell suspensions were also set up with ^{131}I-marked normal human serum which had been absorbed with bovine red cells before iodination.

From Fig. 9 it may be seen that increasing the cell concentration from 0·5 to 4% hardly affects the amount of ^{131}I-marked antibody adsorbed. From

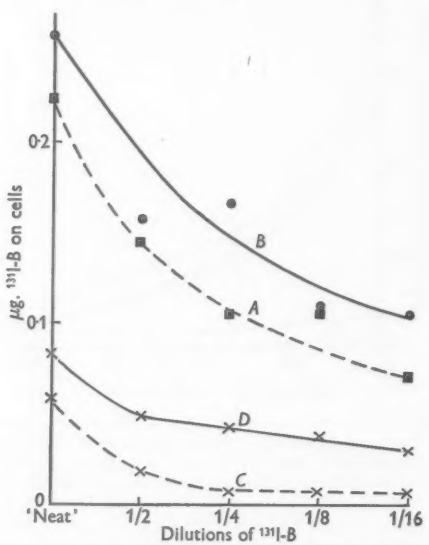


Fig. 8. The use of unmarked serum as diluent for cells and serum to reduce the non-specific adsorption of ^{131}iodinated protein on to human red cells. 'Titrations' of 0·1 ml. ^{131}I-marked incomplete anti-D Rh serum (^{131}I-*B*, 'neat', 2·1% protein) against 0·1 ml. 1% cell suspension. Curves *A* and *C*, Rh-negative cells; curves *B* and *D*, Rh-positive cells. Diluent for cells and ^{131}I-marked serum; curves *A* and *B*, saline; curves *C* and *D*, human serum.

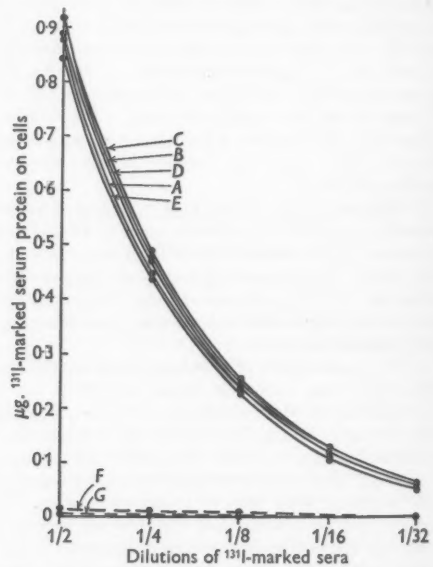


Fig. 9. The effect of the concentration of 'inagglutinable' bovine cells on antibody adsorption. 0·1 ml. ^{131}I-marked infectious mononucleosis serum ('neat', 3·1% protein) with 0·5 ml. cell suspensions in the following concentrations: curve *A*, 4%; curve *B*, 3%; curve *C*, 2%; curve *D*, 1%; curve *E*, 0·5%. Curves *F* and *G*, 0·5 ml. 4% and 0·5% cell suspensions respectively, treated with 0·1 ml. ^{131}I-marked normal human serum ('neat', 2·3% protein).

this it appears that 0·5 ml. of a 0·5 % suspension of bovine cells is capable of absorbing nearly all the antibody present in 0·1 ml. of the 1/2 dilution of the ^{131}I-marked serum sample, a serum whose titre was over 5000. This suggested that the cells have a relatively large number of antigen sites for this antibody.

Expt. C 2. *With* ^{131}I-*marked incomplete anti-D Rh serum* (^{131}I-B). In outline this experiment was similar to Expt. C 1, but instead of controlling any non-specific adsorption by using a ^{131}I-marked normal human serum, the control was effected by 'titrating' ^{131}I-*B* against Rh-negative cells, as well as against Rh-positive cells. The various cell suspensions were made up in compatible A, Rh-negative, heat-inactivated human serum which was also used for the serum dilutions. The antiserum was again used in 0·1 ml. amounts and the cell suspensions in volumes of 0·5 ml.

The results (Fig. 10) differ greatly from those of the previous experiment. The measure of ^{131}I-marked antibody adsorbed increased markedly with the increase in cell concentration. The adsorption on the 1 % suspension of Rh-positive cells was almost exactly twice that on the 0·5 % suspension, but the increase in adsorption with higher concentrations became progressively less.

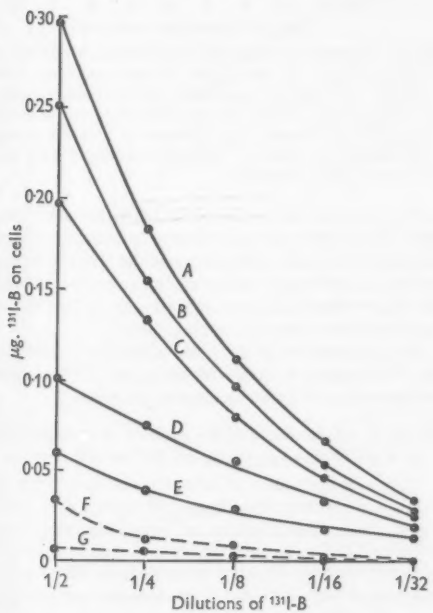


Fig. 10. The effect of the concentration of Rh-positive human cells (O CDe/cDE) on antibody adsorption. 0·1 ml. ^{131}I-marked incomplete anti-D Rh serum (^{131}I-*B*, 'neat', 1·9% protein) 0·5 ml. cell suspensions in the following concentrations: Rh-positive cells, curve *A*, 4 %; curve *B*, 3%; curve *C*, 2%; curve *D*, 1%; curve *E*, 0·5%; Rh-negative cells (O cde/cde), curve *F*, 4%; curve *G*, 0·5%.

With this system therefore it appeared that the number of antigen sites on the positive cells was the factor limiting antibody adsorption. This is in contrast to the findings in the previous experiment in which the concentration of the antibody in the serum appeared to be the limiting factor.

Series D. Estimation of the specific red-cell antibody in a serum

In the two following experiments a known volume of ^{131}I-marked antiserum was repeatedly absorbed with fresh applications of homologous red cells until no further antibody could be removed. The summation of the specifically combined antibody adsorbed on all the cells, expressed as serum protein, was taken as the measure of the original antibody content of the serum.

It is obviously necessary to be assured that no antibody is destroyed during the iodination process. It was shown (p. 748) that the sera used gave the same serological end point before and after iodination, but it is fully realized that ultimately a better quantitative criterion would be required to prove that no antibody had in fact been destroyed. We have given here a value for the total antibody in terms of serum proteins. These values may require subsequent correction when figures are discovered for the relative iodine uptake of the antibodies compared with serum proteins.

Equal volumes of the cell suspension in unmarked compatible serum were placed in a number of tubes which were centrifuged and the supernatant fluid removed. The volume of the ^{131}I-marked serum, whose antibody content was to be determined, was then added to the cell deposit in the first tube and incubated for 20 min. at 37°. The contents of the tubes were centrifuged and the ^{131}I-marked serum transferred to the next tube in the series. The cell deposit in the first tube was washed with one drop of the unmarked diluent serum (cf. p. 749) and the tube recentrifuged. The supernatant drop was then added to the second tube. This second tube was incubated as before and the procedure repeated for as many times as was necessary to exhaust the serum of specific antibody. Finally all the cell deposits were washed three times with saline as usual before they were transferred to the planchets.

Expt. D 1. *Total antibody to bovine red cells in* ^{131}I-*marked infectious mononucleosis serum* (^{131}I-C). 0·5 ml. of a 1/2 dilution of ^{131}I-*C* was repeatedly absorbed with packed cell deposits from 0·5 ml. 0·5 % bovine red-cell suspensions. To control the non-specific adsorption, comparable tests were made with ^{131}I-marked normal human serum (^{131}I-*M*) which had previously been absorbed with bovine red cells. The diluent for cells and the marked sera was inactivated unmarked human serum, also previously absorbed with bovine red cells.

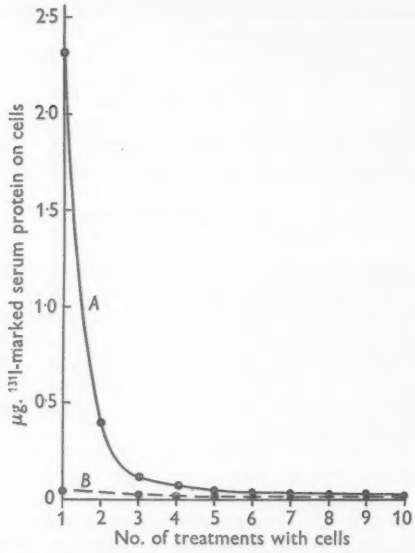


Fig. 11. Assessment of specific red cell antibody content of serum. Curve *A*, absorption of total antibody from 0·5 ml. ^{131}I-marked human infectious mononucleosis serum (protein 1·1 %) by repeated treatment with cell deposits from 0·5 ml. 0·5 % 'inagglutinable' bovine cell suspension. Curve *B*, identical treatment of 0·5 ml. ^{131}I-marked normal human serum (protein, 1·1 %).

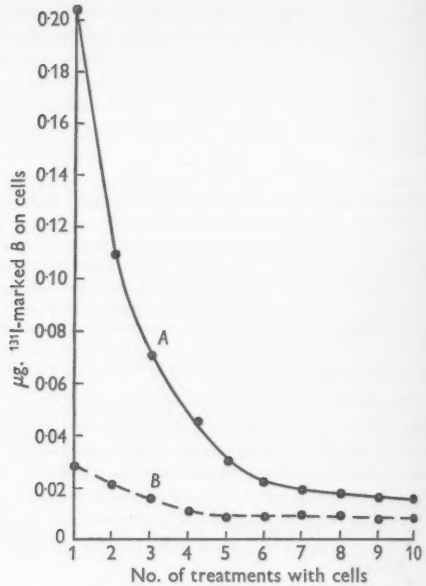


Fig. 12. Assessment of specific red cell antibody content of serum. Curve *A*, absorption of total antibody from 0·5 ml. ^{131}I-marked incomplete anti-D human serum (^{131}I-*B*, protein 0·11 %) by repeated treatment with cell deposits from 1·0 ml. 4 % suspension of Rh-positive cells (A CDe/CDe). Curve *B*, identical treatment with Rh-negative cells (A cde/cde).

The amounts of ^{131}I-marked antibody adsorbed on each sample of cells is shown in Fig. 11. By subtracting the small amount of protein adsorbed from ^{131}I-*M* from that adsorbed from ^{131}I-*C*, a value for the total specific bovine red-cell antibody in the antiserum (in terms of ^{131}I-marked serum protein) could be summated. This amounted to 2·85 μg. in the 0·5 ml. of 1·06 % ^{131}iodinated protein which is equivalent to 38 μg. protein per ml. 7 % protein. It appears that the concentration of ^{131}I-*C* in the sample absorbed is relatively unimportant, for in another experiment in which 0·18 % ^{131}I-*C* was used, a value of 39 μg. protein per ml. 7 % protein was obtained.

The antibody in ^{131}I-*C* was almost completely removed by absorption with boiled bovine red cells, and was left unaffected by boiled guinea pig kidney, illustrating its Paul-Bunnell nature.

Expt. D2. Total incomplete antibodies in ^{131}I-marked Rh serum (^{131}I-B). The same procedure was adopted as in the last experiment. 0·5 ml. of a 1/8 dilution of ^{131}I-*B* was repeatedly absorbed with Rh-positive cells using for each absorption deposits from 1·0 ml. 4 % cell suspension. The figure for the non-specific adsorption was obtained in a comparable test with Rh-negative cells. The diluent for the cells and marked serum was heat-inactivated serum from an A Rh-negative person.

The graph of the results (Fig. 12) is different from that of the previous experiment in that the serum required more absorptions to exhaust it of antibody. In fact, a minute amount of antibody appeared to be left unabsorbed after ten treatments, a fact which could be demonstrated serologically.

The assessment of the total antibody content of the ^{131}I-*B* gave a value of 54 μg./ml. 7 % protein (expressed as ^{131}I-marked serum protein).

Series E. Assessment of the number of antigen sites of a particular specificity on the red-cell surface

If the antigen sites of a particular specificity on a known number of cells are saturated with antibody, then the number of antibody molecules adsorbed divided by the number of cells used should give the average number of functional sites on the individual cell provided that certain assumptions are valid. These are: (i) that a single antigen site combines with a single antibody molecule, and (ii) that all available functional sites are saturated when no further antibody is adsorbed. It is possible that this assumption may be invalidated by steric hindrance which would have the effect of apparently reducing the number of functional sites; this effect

would presumably be greater the larger the number of antigen sites per cell. That very little or no antibody is eluted during the washing process is shown by the results of the washing experiments (Figs. 2 and 3).

In performing the experiments in series E, equal amounts of packed-cell deposits were incubated for 20 min. periods a varying number of times with fresh quantities of the ^{131}I-marked serum. After this treatment each sample of cells was washed with saline as usual and finally transferred to the planchets.

Expt. E 1. *Assessment of the number of Paul-Bunnell antigen sites on a single bovine red cell.* 0·5 ml. amounts of a 0·5% suspension of bovine cells in unmarked human serum (inactivated and previously adsorbed with bovine cells) were placed in each of twelve tubes. The tubes were centrifuged, the supernatant fluid removed and the cell deposits used in the experiment. Four of these cell deposits were treated 1, 3, 5 and 9 times respectively as described above, with 0·1 ml. ^{131}I-*C*, four with ^{131}I-*C* which had been adsorbed with boiled ox cells before iodination, and four with ^{131}I-*M*.

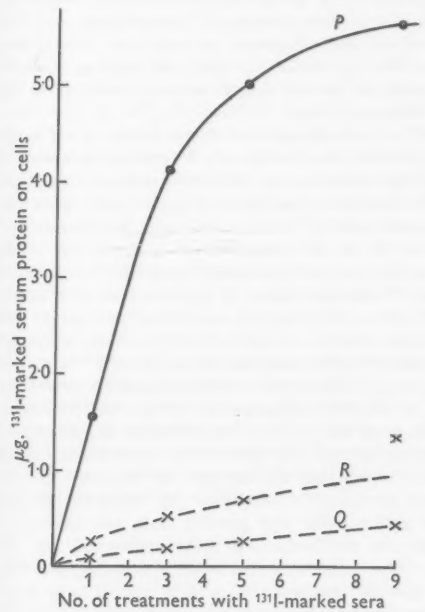


Fig. 13. Assessment of the number of Paul-Bunnell antigen sites on bovine red cells. 0·5 ml. 0·5% 'inagglutinable' bovine cells: curve *P*, repeated treatment with 0·1 ml. quantities of ^{131}I-marked infectious mononucleosis serum (protein, 2·0%); curve *Q*, similar treatment with ^{131}I-marked infectious mononucleosis serum, absorbed with boiled bovine cells before iodination (protein, 1·2%); curve *R*, similar treatment with ^{131}I-marked normal human serum (protein 2·0%).

The graph (Fig. 13) shows the amount of antibody adsorbed after the various treatments. Further experiments, in which the saturation of the cells was assured, gave similar results.

Expt. E 2. *Assessment of the number of D-sites on a single Rh-positive human red cell.* 2% suspensions of Rh-positive and Rh-negative cells in heat-inactivated A_1B Rh-negative serum were prepared. Deposits from 1·0 ml. of these suspensions were repeatedly treated with 0·2 ml. 1/11 dilution of ^{131}I-*Bgl*, 3, 5, 7, 9 and 11 times respectively as previously described. The only antibody in serum *Bgl* reactive against the Rh-positive cells used was anti-D. The results (Fig. 14) show that there is no appreciable increase in antibody adsorption after the seventh treatment with the serum.

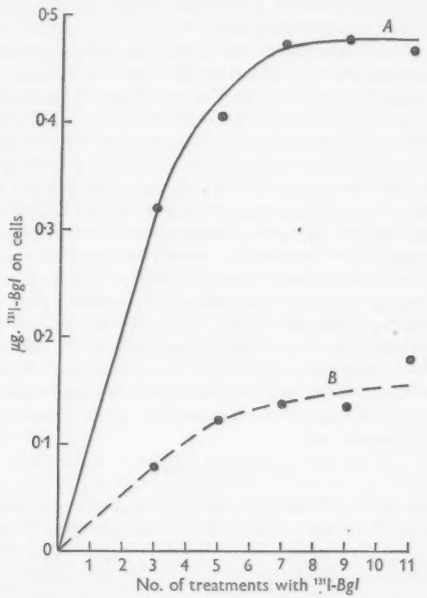


Fig. 14. Assessment of the number of D Rh antigen sites on human red cells. Curve *A*, 1·0 ml. 2% Rh-positive human cells (A_1B CDe/CDe) repeatedly treated with 0·2 ml. quantities of ^{131}I-marked incomplete anti-D human serum (^{131}I-*Bgl*, protein, 0·4%); curve *B*, similar treatment of Rh-negative cells (A_1B cde/cde).

The number of sites on a single red cell can be calculated from the following expression.

$$\frac{P \times N}{\text{Mol.wt.} \times 10^6 \times N \times R},$$

where P = weight (μg.) of antibody adsorbed at saturation,

N = Avogadro's number ($6{\cdot}02 \times 10^{23}$),

Mol.wt. = molecular weight of antibody,

N = number of cells used,

R = ratio antibody I : serum I.

In calculating from this expression the number of sites on a single cell, at present a figure for the molecular weight of the antibody has to be assumed. Deutsch, Alberty, Gosting & Williams (1947) produce evidence that β-haemagglutinins of human plasma represent a small amount of the high molecular weight portion of the γ_1-globulin. Davis, Moore, Kabat & Harris (1945) show that the Wassermann antibody is associated with both the light and heavy components of whole syphilitic serum ($S = 7$ and 19 respectively). Kabat (1939), in studies on pneumococcal antibodies, states that whereas in cow, horse and pig these have a molecular weight of $9{\cdot}9 \times 10^5$, in human, rabbit and monkey the figure is $1{\cdot}6$–$1{\cdot}8 \times 10^5$.

If it is assumed that there are no differences between the molecular weights and the ratios of iodination of the two antibodies, then calculation of the number of sites per cell for the bovine and human cells (A_1B CDe/CDe) gives figures of 5×10^5 and $5{\cdot}5 \times 10^3$ respectively (assuming a molecular weight of $1{\cdot}65 \times 10^5$ and a ratio of iodination (R) of 0·9). As both the molecular weight and the ratios of iodination are at present unknown, it is necessary to repeat the calculation with assumed possible molecular weights and iodination ratios which produce the least difference in the number of sites on the two systems. Thus, changing the molecular weight of the Paul-Bunnell antibody to 1×10^6 and the iodination ratio (R) of the incomplete D Rh antibody to 0·3, the number of Paul-Bunnell sites on the bovine cell is still 5 times that of the D sites on the human cell.

DISCUSSION

The use of ^{131}I as a marker for antibody molecules appears to have many distinct advantages. If the amount of iodine introduced is kept below two iodine atoms per protein molecule the combining capacity of the antibody is apparently not interfered with, and certainly with the two single antibody systems studied no reduction in the serological titre of the antibody could be demonstrated. Even with a greater amount of introduced iodine the reduction in the serological titre was slight.

Although at first sight it might appear preferable to use the ^{131}I in a carrier-free form, this procedure was not adopted for a number of reasons. If carrier-free ^{131}I is used, then the introduction of a quantity of radioactivity of the order of 1 mc into 1 ml. antiserum would mean that only a very small proportion of the total number of antibody molecules would have an opportunity of combining with the iodine. It is the behaviour of these ^{131}I-marked antibody molecules that is studied by measurement of the radioactivity. If there are any slight differences between individual molecules, e.g. in serological behaviour, it is not impossible that these differences might also influence the relative uptake of ^{131}I where the amount of this is strictly limited by competition with other molecules. If in addition there are slight differences in the serological behaviour of ^{131}iodinated and uniodinated antibody molecules the same argument would apply when the combination with cells of these two types of antibody molecule is considered.

If enough carrier iodine is added to give an average uptake of about one atom per protein molecule, then any serological differences introduced thereby are to a large extent common to all the antibody molecules, and the possibility of selective competition is reduced, if not eliminated entirely. It is assumed that the difference between a ^{131}I- and a ^{127}I-marked antibody is altogether too small to detect by the methods employed, and in any case is likely to be far less than the possible difference between a ^{131}I-marked antibody molecule and an unmarked one.

The method of introducing the iodine into the serum proteins which was selected involved the sacrifice of a proportion of the ^{131}I, which for various reasons remained uncombined with the protein. Nevertheless, it was considered more satisfactory to do this than risk creating chemical conditions which might cause alteration to the protein molecules.

The first obstacle which presented itself in the attempt to measure specific ^{131}I-marked antibody on cellular antigens was the non-specific adsorption of ^{131}I-marked serum proteins by the cells. This non-specific adsorption was reduced, as described in series B, by the pretreatment of the cells with unmarked compatible serum before incubation with the ^{131}I-marked sera. It appears that cells always adsorb a certain amount of serum protein on their surface and, by treating them with the unmarked serum first, the amount of non-specific ^{131}I-marked serum adsorbed was reduced to such an extent that it no longer overshadowed the specific ^{131}I-marked antibody adsorption. Nevertheless, the absorption of non-specific material was of considerable interest. Thus, although the amount of human γ-globulin non-specifically adsorbed by Rh-positive and -negative cells was greater than the amount of specific antibody γ-globulin adsorbed by Rh-positive cells, only this latter specifically adsorbed γ-globulin caused the cells to agglutinate in the presence of rabbit anti-human globulin serum.

From the serological aspect, the use of the isotope technique affords a means of establishing the amount of antibody to cellular antigens in a serum and also the number of particular antigen sites per cell. These assessments would be very difficult by any other known method. With cellular antigens, the estimation of small amounts of antibody sensitization by the Kjeldahl technique is not satis-

factory. Kabat & Mayer (1948), in discussing the estimation of antibodies to intact red cells by the Kjeldahl method, state that 'with intact erythrocytes... the amount of antibody N removed is so small a fraction of the erythrocyte N that the method is impracticable'.

It may appear that the amount of antibody protein per ml. serum estimated in both the infectious mononucleosis serum and the Rh antiserum was very small in relation to the respective serological titres. However, it should be remembered that probably single antigen-antibody systems were being measured and not total antibody against all the antigens on a red cell.

The estimation of the number of sites of a particular antigen on a red cell is important because this is obviously a factor which must influence the agglutination of the cells by the corresponding antibody. The finding that the number of Paul-Bunnell sites is considerably greater than the number of D-sites on a human Rh-positive cell (CDe/CDe) offers a reasonable explanation of certain phenomena observed empirically when carrying out serological tests on these two systems. For instance, it is very easy to absorb completely the Paul-Bunnell antibody from a serum and relatively few bovine cells are required. On the other hand, it is much more difficult to absorb the D Rh antibody from a serum and a much larger number of Rh-positive cells is needed. The frailness of the agglutination due to agglutinating Rh antibodies may also be a reflection of the relatively small number of D Rh antigen sites on the cells. With few antigen sites there can be only a small number of antibody molecules binding together the areas of adjacent cells which are in contact in an agglutinate. Finally the relatively few D Rh antigen sites may well account for the very small change in the surface charge of Rh-positive cells when strongly sensitized with Rh antibody (Coombs & Race, 1945).

The authors intend to apply this method to the estimation of the relative number of antigen sites C, D, E, c and e on human red cells. The assessment of the D sites on homozygous (DD) and heterozygous (Dd) cells will also be of great interest.

SUMMARY

1. Experiments have been carried out with antisera marked with ^{131}I in an attempt to study quantitatively the interaction of serum antibodies and red cell antigens.

2. Studies were made on inagglutinable systems. These were 'inagglutinable' bovine cells with ^{131}I-marked rabbit anti-bovine red cell and ^{131}I-marked human infectious mononucleosis sera, and also human cells with ^{131}I-marked human Rh incomplete anti-D sera.

3. The complication due to the non-specific adsorption which interfered with quantitative determinations was avoided by pretreatment of the cells with unmarked serologically compatible serum.

4. The ratio of iodine uptake by the γ-globulin fraction of serum to that of the whole serum was found to pass through a maximum (0·92) with an iodine content of about one atom per protein molecule (assumed average mol.wt. of 10^5).

5. The amount of Paul-Bunnell antibody (in terms of ^{131}I-marked serum protein) in an infectious mononucleosis serum was found to be about 40 μg./ml. serum (7% protein). In the same terms, the amount of D-antibody in an incomplete Rh antiserum was estimated at 54 μg.

6. The number of Paul-Bunnell sites on a single bovine red cell was found to be considerably greater than the number of D sites on a human Rh-positive cell (CDe/CDe). The implication of these results is discussed in the light of some observed serological phenomena.

The authors would like to express their thanks to the Agricultural Research Council for grants to two of them (J. C. B. and V. R.) and for an expenses grant, to Mr G. Wells and his colleagues in the Isotope Division, Atomic Energy Research Establishment, for their unfailing kindness in supplying the ^{131}I, and to Dr J. Schulman for a gift of polyvinyl alcohol. They are also indebted to Dr C. B. V. Walker and Dr C. Cameron of the Cambridge Regional Blood Transfusion Service for their help in supplying red cells and sera, and to Dr S. G. J. Hayhoe, Dr D. Robertson Smith, Mr B. W. Gurner, Mr R. J. Flemans and Miss C. M. Pegg of the Department of Medicine in this University for performing numerous venepunctures and blood counts.

REFERENCES

Cohen, S. (1951). *J. Immunol.* **67**, 339.
Cohn, E. J., McMeekin, T. L., Oncley, J. L., Newell, J. M. & Hughes, W. L. (1940). *J. Amer. chem. Soc.* **62**, 3386.
Coombs, R. R. A., Mourant, A. E. & Race, R. R. (1945). *Brit. J. exp. Path.* **26**, 255.
Coombs, R. R. A. & Race, R. R. (1945). *Nature, Lond.*, **156**, 233.
Dacie, J. V. (1950). *Nature, Lond.*, **166**, 36.
Davis, B. D., Moore, D. M., Kabat, E. A. & Harris, A. (1945). *J. Immunol.* **50**, 1.
Deutsch, H. F., Alberty, R. A., Gosting, L. J. & Williams, J. W. (1947). *J. Immunol.* **56**, 183.
Francis, G. E., Mulligan, W. & Wormall, A. (1951). *Nature, Lond.*, **167**, 748.
Gleeson-White, M. H., Heard, D. H., Mynors, L. S. & Coombs, R. R. A. (1950). *Brit. J. exp. Path.* **31**, 321.
Kabat, E. A. (1939). *J. exp. Med.* **69**, 103.
Kabat, E. A. & Mayer, M. M. (1948). *Experimental Immunochemistry*, p. 77. Springfield, Illinois: Charles C. Thomas.
Kekwick, R. A. (1940). *Biochem. J.* **34**, 1248.
Masouredis, S. P., Melcher, L. R. & Koblick, D. C. (1951). *J. Immunol.* **66**, 297.
Pressman, D. & Sternberger, L. A. (1950). *J. Amer. chem. Soc.* **72**, 2226.
Race, R. R. (1944). *Nature, Lond.*, **153**, 771.
Race, R. R. & Sanger, R. (1950). *Blood Groups in Man*, p. 82. Oxford: Blackwell.
Wintrobe, M. M. (1942). *Clinical Hematology*, p. 200. London: Henry Kimpton.
Wiener, A. S. (1944). *Proc. Soc. exp. Biol., N.Y.*, **56**, 173.

The Chemical Composition of the Soluble and Insoluble Fractions of the Bacterial Cell

By W. R. FERNELL and H. K. KING
Department of Biochemistry, University of Liverpool

(*Received* 27 *November* 1952)

Investigation of the chemistry of the bacterial cell wall has been retarded by lack of suitable methods. The results of immunological investigations have suggested that in some organisms its structure is complex, with successive layers of different antigens. Another approach has been through the extraction of whole-cell preparations with organic solvents. Morgan & Partridge (1940) and Freeman, Challinor & Wilson (1940) have extracted polysaccharide-protein complexes from certain Gram-negative bacteria. These substances accounted for about 10% of the whole cell and were believed on immunological evidence to comprise the outer layers. Henry & Stacey (1946) extracted Gram-positive bacteria with bile salt and obtained a magnesium ribonucleate which they considered to be a surface component and responsible for the retention of the Gram stain. Caution must be exercised in interpreting the results of these extraction experiments since it is difficult to establish from which part of the cell the extracted material has come.

Curran & Evans (1942) and King & Alexander (1948) killed bacteria by prolonged shaking with minute glass beads. Dawson (1949) showed that this treatment ruptures the cell wall and allows the contents to escape, leaving the empty shell more or less intact. This seemed to offer the possibility of separating cell walls for chemical study. Salton & Horne (1951*a*, *b*) separated cell-wall material by this method, and also by heat treatment and by treatment with alkali. More recently, Salton (1952*a*) has found that the cell wall of *Streptococcus faecalis* is composed of a protein-polysaccharide complex, similar, perhaps, to those obtained by extraction methods from the Gram-negative bacteria (Morgan & Partridge, 1940; Freeman *et al.* 1940). He suggests that mucoproteins may be important components of bacterial cell walls. Salton (1952*b*) finds that the lipid content of Gram-positive cell walls is low (1–2%) compared with that of Gram-negatives (20%), and there are also differences in the amino acids of the protein component. Mitchell & Moyle (1951) find that three-quarters of the cell-wall material of *Micrococcus pyogenes* is accounted for by a glycerophosphoprotein complex which they regard as being probably characteristic of Gram-positive types. Holdsworth (1952) finds that the 'insoluble fraction' of *Corynebacterium diphtheriae* contains 60% protein and 25% carbohydrate. Northcote & Horne (1952) have described certain polysaccharides from yeast cell walls.

METHODS

Organisms. Four Gram-negative types were studied (*Escherichia coli* E106; *Pasteurella pseudotuberculosis* N.C.T.C. 1102; *Proteus vulgaris* L10; *Pseudomonas aeruginosa* L15): three Gram-positive types (*Bacillus subtilis* E4; *Micrococcus pyogenes*, var. *albus* (*Staphylococcus albus*) L14; *Sarcina lutea* L21): and one acid-fast organism (*Mycobacterium phlei*, N.C.T.C. 525). *Myco. phlei* and *Past. pseudotuberculosis* were obtained from the National Collection of Type Cultures: the other organisms were stock laboratory strains and the numbers refer to our own list.

Media. Glucose broth: a peptone-Lemco-yeast extract broth (Oxo Dried Medium. CM1, Oxo Ltd., Thames House, London, E.C. 4), with addition of 0·5% glucose. The Oxo medium was made up in the appropriate volume of tap water and sterilized (5 lb./30 min.). The glucose was prepared as an acidified 50% (w/v) solution, sterilized (5 lb./30 min.) and the appropriate volume added aseptically to the broth.

Glucose agar: glucose broth solidified with 2% B.D.H. agar powder.

Stock cultures were maintained on glucose-agar. For each experiment five Roux bottles, each containing 150 ml. glucose agar, were inoculated with 5 ml. of a 24 hr. glucose-broth culture of the organism under examination. After incubating for 19 hr. at 37° the organisms were washed off with water, passed through a coarse sintered-glass filter to remove particles of agar, centrifuged, washed three times on the centrifuge, and suspended in 10 ml. water. The yield was about 0·5 g. dry weight in most cases. The organisms were 'mature', i.e. they had been harvested shortly after completion of the phase of logarithmic growth.

Disintegration of organisms

The 10 ml. suspension was placed in a 1 oz. screw-cap bottle with 10 g. of grade 12 Ballotini (Chance Bros., Smethwick, England); the bottle was shaken for 4 hr. along its long axis, at about 750 strokes/min. with a 2·5 in. stroke in a machine similar to that used by King & Alexander (1948). The bottles were packed in ice during shaking, and the temperature of the contents did not exceed 10°. The shaker accommodated two bottles and all runs were done in duplicate. Viable counts were made before and after shaking, and in all cases a reduction to less than 10^{-4} of the original value was obtained. Microscopic examinations were also made. With *B. subtilis*, for example, the unshaken organisms were clearly visible under dark-ground illumination and highly refractile. After 0·5 hr. shaking, most of the cells appeared to be broken; usually the end of the bacillus seemed to have been broken off. The broken cells were also easily distinguished as they were not highly refractile. Some cells still retained the Gram stain: the remainder stained only weakly. After 1 hr., when the viable count had already, in this case, fallen below 10^{-4} times its initial value, there were very few intact cells and an appreciable amount of amorphous debris in the form of particles on the limit of visibility of the dark-ground microscope. The Gram staining reaction was almost negative. Further shaking led to an increase in the amount of debris. These observations were made on the bulk of the fluid in the vessel: the froth which formed during shaking contained rather more intact cells.

After shaking, the disintegrated material was poured through a coarse (grade 1) sintered-glass filter, which separated off the Ballotini. The Ballotini were then washed on the filter with 5 ml. lots of water until the runnings were quite clear. The total volume (material plus washings) was now 30–40 ml. Harvesting and disintegration was a full day's work and the material was frozen solid at this stage and left overnight. After thawing, the disintegrate was centrifuged for 10–15 min. at 10000 rev./min. ($14000 \times g$) to yield an almost clear supernatant. This was carefully decanted off and the insoluble fraction was resuspended in 15–20 ml. water with very thorough shaking, and recentrifuged. This washing procedure was carried out three times: the absence of any frothing in the supernatant then showed that no more soluble protein material was being washed out. The washings were combined with the original supernatant and made up to 100 ml. This was the 'soluble fraction' of the cell. The insoluble material was suspended in 20 ml. water and a homogeneous suspension obtained by vigorous mechanical shaking. Any extraneous matter was removed by passing through a coarse sintered-glass filter, and the filter washed, and the runnings made up to a total volume of 25 ml. This was the 'insoluble fraction'. During all centrifuging procedures the centrifuge cups were sealed with Sellotape: otherwise sufficient foreign matter found its way in to cause serious errors.

Analytical methods

Dry weight. This was determined by freeze-drying a suitable sample (5 ml. insoluble or 25 ml. soluble fraction).

Ash content. A suitable volume of the solution or suspension was freeze-dried in a nickel crucible and incinerated at 600° in an electric muffle furnace. Errors in ash content arising from abrasion of the beads were small. In one experiment 490 mg. (dry weight) of organism yielded 42·5 mg. ash before disintegration, 43 mg. after treatment. The increase in ash content was thus within the experimental error of determination. On the other hand, very serious errors in ash content may arise if the centrifuge cups are not covered during centrifugation.

Total nitrogen by Kjeldahl. The incineration technique of Chibnall, Rees & Williams (1943) and the distillation apparatus of Markham (1942) were used.

Phosphate. Phosphate was determined by the method of Berenblum & Chain (1938).

Hexose and pentose. These were estimated simultaneously by the differential colorimetric method of Fernell & King (1953).

Deoxypentose. This was determined by Dische's (1930) method. The reaction could not be carried out with deeply coloured materials such as the insoluble fraction of *Ps. aeruginosa*. The insoluble fractions of other organisms also gave trouble on account of turbidity. This was overcome as follows. The material was heated with the Dische reagent in the usual way, and then filtered through a very thin layer of washed talc supported on a fine (5/3) sintered-glass filter. The talc did not remove any of the colour, nor were the fine particles filtered off by the talc coloured. As no deoxyribose was available for use as a standard, a commercial sample of thymus nucleic acid was used; it gave correct analytical figures for N and P, and was free of pentose. This was convenient, since it was assumed that the deoxyribose present would be in the form of deoxyribonucleic acid (DNA), and results are expressed in terms of DNA.

Total nucleic acid. Mitchell (1950) describes a method for the spectroscopic estimation of nucleic acid in bacteria based on the work of Malmgren & Heden (1947). The absorption of a suspension is measured at 260 mμ. and a correction applied for scattering. A difficulty arises in the selection of the correct factor for the absorption coefficient. Mitchell takes $E^{1\%}_{1\,cm.}$ 200, which he regards however as a minimum value. A figure of 250 accorded better with our own measurements on both thumus and yeast mucleic acids. We found no change in the specific absorption at 260 mμ. when organisms were disintegrated, and concluded that the absorption coefficient does not change when nucleic acids are liberated from the cell. Although, as Mitchell admits, 'the method depends on certain assumptions of questionable validity', it is valuable for making comparative observations such as those with which the present study is concerned. We found it unsuitable however for the insoluble fractions. Absorption, or scattering, rises very rapidly as the wavelength decreases. Some organisms show no break at all at 260 mμ., others only a slight inflexion. Whether the steep rise observed at the shorter wavelengths is due to non-specific absorption or to anomalous scattering we do not know. Mitchell's correction for scattering is based on the assumption that the scattering particles were bacterial cells, and is not necessarily valid when dealing with smaller particles of very varying size.

RESULTS

The possibility of changes in the cell constituents during treatment was considered, although the temperature was kept below 10° during disintegration. A suspension of *Esch. coli* was therefore disintegrated and samples withdrawn at intervals for assay. Values for nucleic acid, deoxypentose, pentose, and hexose did not differ significantly from those for the untreated organism. The absorption spectrum revealed a considerable fall in scattering in the first half-hour but no change in the specific nucleic acid absorption.

Composition of fractions

Two suspensions, prepared from separate cultures of each organism, were disintegrated and assayed separately. The two series of replicate results are listed under 'culture 1' and 'culture 2' in the tables. The proportion of the whole cell accounted for by the insoluble fraction is given in Table 1, and the analytical figures for the separate fractions in Table 2.

Table 1. *Insoluble fraction of disintegrated bacteria expressed as percentage dry weight of whole organism*

	Culture 1	Culture 2
B. subtilis	53·7	54·7
Sarc. lutea	45·9	47·6
M. pyogenes	40·5	41·6
Esch. coli	37·1	39·6
Ps. aeruginosa	76·6	78·0
Pr. vulgaris	29·4	20·8
Past. pseudotuberculosis	64·1	65·5
Myco. phlei	51·9	51·7

Table 2. *Analysis of insoluble and soluble fractions of disintegrated bacteria*

(All results expressed as percentage dry weight of fraction concerned.)

		B. subtilis		*Sarc. lutea*		*M. pyogenes*		*Esch. coli*		*Ps. aeruginosa*		*Pr. vulgaris*		*Past. pseudo-tuberculosis*		*Myco. phlei*	
		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Ash	Insoluble	3·7	3·9	10·0	10·7	8·4	7·7	8·0	8·5	3·60	3·72	8·5	7·9	3·6	3·3	9·8	10·3
	Soluble	12·5	12·5	12·5	11·2	14·8	13·2	7·9	7·9	7·8	7·2	8·0	7·9	7·3	7·1	7·8	8·1
Total N	Insoluble	8·7	8·7	6·1	5·6	9·9	10·1	9·5	9·6	10·0	10·3	9·6	9·4	5·6	5·8	6·9	6·3
	Soluble	7·9	7·6	8·0	8·9	11·0	10·0	12·9	12·2	9·3	9·3	11·3	11·1	7·8	7·9	7·7	8·0
Total P	Insoluble	0·47	0·44	0·28	0·19	1·74	1·76	0·85	0·85	0·99	1·02	0·37	0·34	0·22	0·23	0·14	0·15
	Soluble	0·65	0·65	1·67	1·63	2·4	2·1	1·93	2·00	3·0	2·9	0·96	0·93	1·80	1·75	1·26	1·30
Hexose	Insoluble	2·58	2·41	10·9	10·2	3·8	3·7	10·3	10·9	5·0	6·8	7·7	7·8	2·33	1·80	4·7	4·8
	Soluble	15·1	16·1	5·9	8·0	6·0	5·0	10·2	11·6	6·6	6·6	3·1	2·9	14·8	14·4	6·4	6·2
Pentose	Insoluble	0·85	0·85	0·95	0·90	0·95	1·24	1·50	1·39	2·3	2·0	1·74	1·83	0·14	0·14	4·4	3·3
	Soluble	3·8	4·1	1·72	1·70	7·7	6·5	4·4	4·2	4·3	4·4	3·7	3·4	3·1	2·9	6·4	6·2
Deoxyribonucleic acid	Insoluble	0·28	0·28	0·39	0·33	0·26	0·26	1·75	1·81	(Not assayed)		1·28	1·28	0·69	0·75	0·77	0·78
	Soluble	1·26	1·24	1·96	1·98	2·78	2·35	5·07	5·25	14·1	14·3	6·0	6·4	2·32	2·05	7·6	7·8
Total nucleic acid	Soluble	16·1	16·9	11·8	11·7	15·1	12·8	20·3	20·7	25·8	25·8	20·0	18·6	19·6	17·3	16·4	15·9

The ash content of the soluble fraction is higher in the Gram-positive than in the Gram-negative organisms. In all cases the soluble fraction is richer in phosphorus than the insoluble fraction.

Carbohydrates

The pentose content is higher, usually much higher, in the soluble fraction. This is probably due to the very low nucleic acid content of the insoluble material. Owing to the stability of the pyrimidine nucleotides to acid hydrolysis (Schneider, 1945) only 45% of the ribonucleic acid pentose will be estimated. In the soluble fraction which has a high nucleic acid content, the figures given may be only half the true value. The precise correction to be applied cannot be stated since no detailed analysis of the nucleic acids was made. 'Pentose' does not include deoxypentose. Amino sugars do not assay in the methods used.

Holdsworth (1952) found 2·0% pentose in the soluble fraction of *C. diphtheriae* and 9·7% in the insoluble fraction, but the cell wall of this organism contains a polysaccharide with an arabinose unit.

The three Gram-positives are relatively poor in deoxypentose, especially in the insoluble fraction.

Nucleic acid

Data are given for the soluble fraction only. Absorption spectra of both fractions were examined in all cases. In the insoluble fractions scattering was high and the photometer reading increased rapidly as wavelength decreased, with either no detectable break in the curve at 260 mμ. or at most only a slight inflexion. Even when scattering is reduced by hydrolysis with acid, the spectra still suggest that no appreciable amounts of nucleic acid are present. This is in agreement with Salton & Horne's (1951*b*) findings for the cell wall of *Strep. faecalis*. The soluble fractions, on the other hand,

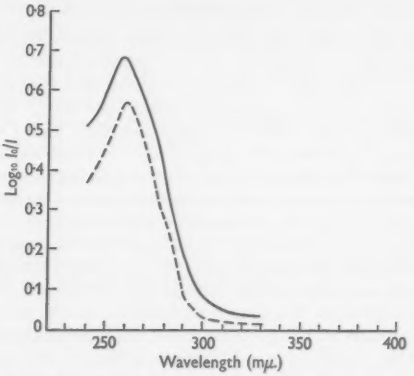


Fig. 1. Absorption spectra of soluble fractions; *Esch. coli*, 0·120 mg./ml. (continuous line); *B. subtilis*, 0·136 mg./ml. (broken line). Measured in 1 cm. cell in Beckman spectrophotometer.

gave absorption spectra characteristic of nucleic acid. Fig. 1 gives the curves obtained with *B. subtilis* and *Esch. coli*; the other organisms gave curves similar in form but differing in the height of the 260 mμ. peak.

Effect of medium

The composition of a bacterial cell may be much influenced by the medium in which it is grown. No detailed investigations were made of the relative effect of the medium on the two fractions, but in one experiment we compared the composition of the fractions from cultures of *B. subtilis* and *Esch. coli* grown both on our standard glucose-agar medium, and on a similar medium without glucose.

The results (Table 3) show that on the whole the soluble fraction is more affected by this change in the medium.

Table 3. *Influence of medium on composition of fractions*

(*Esch. coli* and *B. subtilis* were grown on (*a*) plain agar and (*b*) 0·5% glucose agar. The table gives the yield of the insoluble fractions expressed as % dry wt. of the whole cell and the components of the various fractions as % dry wt. of the fraction concerned.)

	Esch. coli		*B. subtilis*	
	Glucose	No glucose	Glucose	No glucose
Insoluble fraction (% dry wt. of whole cell)	42	37	55	43
N, insoluble fraction	9·5	8·8	8·8	12·6
N, soluble fraction	12·5	11	7·5	10
P, insoluble fraction	0·84	1·28	0·45	0·45
P, soluble fraction	1·94	1·84	0·54	1·84
Pentose, insoluble fraction	1·5	1·5	0·9	0·7
Pentose, soluble fraction	4·6	1·9	4·0	2·5
Hexose, insoluble fraction	10·6	11·9	2·5	5·4
Hexose, soluble fraction	10·9	2·3	15·8	1·2
Deoxyribonucleic acid, insoluble fraction	1·75	2·42	0·28	1·04
Deoxyribonucleic acid, soluble fraction	5·6	2·64	1·3	1·48
Total nucleic acid, soluble fraction	20·4	16·4	16·4	12·6

DISCUSSION

Cooper, Rowley & Dawson (1949) criticize the disintegration technique on the grounds that up to 7 % of the cells escape destruction, and Salton & Horne (1951*b*) claim only that 'over 90 %' were disintegrated. Mitchell & Moyle (1951) found less than 1 % of cells of *M. pyogenes* intact. These authors all used the Mickle disintegrator with a higher speed but a much shorter stroke than our shaker. Previous work (King & Alexander, 1948) showed that appropriate shaking technique would destroy nearly all the cells, and King & Stein (1950) achieved complete sterilization of even thick suspensions of *B. anthracis*. We found, in addition to the factors discussed by King & Alexander, that the proportion of the vessel occupied by the suspension plus beads is important. Destruction is much retarded if an appreciable air space is not left in the vessel (Fig. 2).

Dawson (1949), Salton & Horne (1951*b*) and Mitchell & Moyle (1951) have examined, by electron microscopy, materials corresponding to our 'insoluble fraction'. They observed no formed elements other than those derived from the cell wall. Mitchell & Moyle obtained two fractions on centrifugation of their disintegrated bacteria, a lower layer containing only recognizable cell-wall fragments and an upper layer containing finer particles as well. They conclude, however, that the latter (in view of their chemical composition) were also derived from the cell wall. Our material includes both their fractions. Although we have not been able to submit our insoluble fraction to the final test of electron microscopy, it is clear that it is derived mainly, if not entirely, from the cell wall. The cell wall has hitherto been generally regarded as a relatively thin outer layer, by analogy with the cell wall of plants. It is therefore surprising to find that the insoluble fraction comprises so large a portion of the whole cell. Nevertheless, this agrees with the findings of others, e.g. 25 % for *M. pyogenes* (Mitchell & Moyle, 1951); 57 % for *C. diphtheriae* (Holdsworth, 1952). In a coccus, say, 0·8 μ. in diameter, a wall only 80 mμ. thick would occupy half the volume: Dawson (1949) obtained a value of 15–20 mμ. by direct measurement of a cell-wall preparation of *M. pyogenes* which had however been subjected to the intensive drying required for electron microscopy. Evidently, the cell wall separated by the disintegration procedure, i.e. our insoluble fraction, represents a substantial outer zone of the cell rather than the classical idea of a cell wall, and the term cell wall may require redefinition as far as bacteria are concerned.

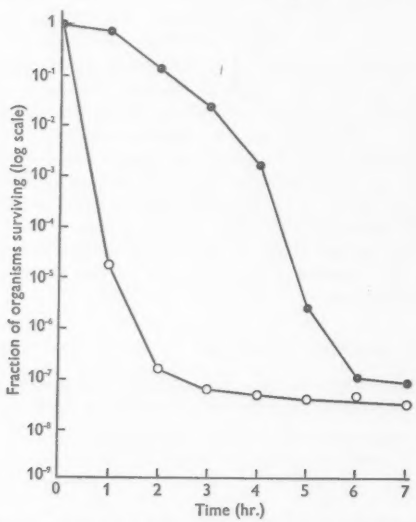


Fig. 2. Effect of volume of contents of vessel on disintegration of organisms. A thick suspension of *Esch. coli* was shaken in a 1 oz. bottle completely filled with the suspension and beads (●—●) and also in a bottle only half filled (○—○). Samples (0·5 ml.) were withdrawn at hourly intervals and the viable count determined. With the full bottle the rate of destruction was relatively low until, after 4 hr., sufficient fluid had been withdrawn for the purpose of making the counts to leave an appreciable air space in the vessel.

The most striking difference between the insoluble, or outer-zone fraction, and the soluble fraction is the paucity of nucleic acid in the former. The characteristic absorption at 260 mμ. is absent and the figures for phosphorus, pentose and deoxypentose are lower (in most cases much lower) than those for the soluble fraction. Apart from this, the chemical composition of the insoluble fraction shows no clear pattern common to all species. This is hardly surprising in view of the variety of materials reported as characteristic of the cell wall. Two quite different substances have indeed been claimed as the characteristic surface component of the Gram-positive group, namely, the magnesium ribonucleate of Henry & Stacey (1946) and the polyolphosphate, or phosphate ester of an unidentified polyhydric alcohol, of Mitchell & Moyle (1951). Henry & Stacey, however, did not demonstrate unequivocally that their material was derived from the cell wall, whilst Mitchell & Moyle worked with *M. pyogenes*, an organism whose insoluble fraction is exceptionally rich in phosphorus. Holdsworth (1952) found that 45 % of the cell wall of the Gram-positive *C. diphtheriae* consisted of an oligosaccharide-protein complex. The mucoprotein described by Salton (1952*a*) in *Strep. faecalis*, like the similar substances from Gram-negative types (Morgan & Partridge, 1940; Freeman *et al.* 1940) cannot be the main cell-wall component as it accounts for less than 10 % of the whole cell.

SUMMARY

1. The soluble and insoluble fractions of eight species of bacteria were separated by centrifuging the material obtained after disintegrating the cells by shaking with glass beads.

2. The insoluble material, believed to be at least mainly derived from the cell wall, accounted in most cases for about half of the dry weight of the cell.

3. Estimations were performed on both fractions for ash, nitrogen, phosphorus, hexose, pentose, deoxypentose and nucleic acid, the last being determined spectroscopically.

4. Wide variations were found among different organisms but certain regularities were observed. Notably, nucleic acid was confined to the soluble fraction, which was also much richer in phosphorus, pentose and deoxypentose.

5. Concepts of the bacterial cell wall are discussed in the light of these findings.

We are deeply indebted to Prof. R. A. Morton, F.R.S., for his interest and encouragement throughout; to the Ministry of Education for a grant to one of us (W.R.F.) under the Further Education and Training Scheme; and to the Medical Research Council for financial assistance in the provision of materials and equipment.

REFERENCES

Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Cooper, P. D., Rowley, D. & Dawson, I. M. (1949). *Nature, Lond.*, **164**, 842.
Curran, H. R. & Evans, F. R. (1942). *J. Bact.* **43**, 125.
Dawson, I. D. (1949). In Miles, A. A. & Pirie, N. W., *The Nature of the Bacterial Cell Surface.* Oxford: Blackwell Scientific Publications.
Dische, Z. (1930). *Mikrochemie*, **8**, 4.
Fernell, R. W. & King, H. K. (1953). *Analyst*, **78**, 80.
Freeman, G. G., Challinor, S. W. & Wilson, J. (1940). *Biochem. J.* **34**, 307.
Henry, H. & Stacey, M. (1946). *Proc. Roy. Soc.* B, **133**, 391.
Holdsworth, E. S. (1952). *Biochim. biophys. Acta*, **9**, 19.
King, H. K. & Alexander, H. (1948). *J. gen. Microbiol.* **2**, 315.
King, H. K. & Stein, J. (1950). *J. gen. Microbiol.* **4**, 48.
Malmgren, B. & Heden, C. (1947). *Acta path. microbiol. scand.* **24**, 417.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Mitchell, P. (1950). *J. gen. Microbiol.* **4**, 399.
Mitchell, P. & Moyle, J. (1951). *J. gen. Microbiol.* **5**, 981.
Morgan, W. T. J. & Partridge, S. M. (1940). *Biochem. J.* **34**, 169.
Northcote, D. H. & Horne, R. W. (1952). *Biochem. J.* **51**, 232.
Salton, M. J. R. (1952*a*). *Biochim. biophys. Acta*, **8**, 510.
Salton, M. J. R. (1952*b*). *Biochim. biophys. Acta*, **9**, 334.
Salton, M. J. R. & Horne, R. W. (1951*a*). *Biochim. biophys. Acta*, **7**, 19.
Salton, M. J. R. & Horne, R. W. (1951*b*). *Biochim. biophys. Acta*, **7**, 177.
Schneider, W. C. (1945). *J. biol. Chem.* **161**, 293.

The Mechanism of Inhibition of Cholinesterases by Organophosphorus Compounds

By W. N. ALDRIDGE and A. N. DAVISON
Medical Research Council Unit for Research in Toxicology, Serum Institute, Carshalton, Surrey

(*Received 7 April* 1953)

In a previous paper it was concluded that organophosphorus compounds inhibit cholinesterase by phosphorylating the enzyme and a chemical mechanism was suggested for both inhibition and reversal of inhibition (Aldridge, 1953*a*). A general formula of organophosphorus inhibitors may be represented as $(R)_2(X)PO$ where X is the group labile to hydrolysis and R is usually an alkoxy group. According to the phosphorylation theory, provided the R group is maintained constant, even though the X group is varied, the same inhibited enzyme must result. Wilson (1952) has shown that the inhibition of electric-eel cholinesterase after both tetraethyl pyrophosphate and diethyl phosphorofluoridate reverses at the same extremely slow rate and is also increased to the same degree by the presence of high concentrations of choline.

True cholinesterase activity returns quickly after inhibition by dimethyl *p*-nitrophenyl phosphate (Aldridge, 1953*a*) and the inhibition of rat pseudo cholinesterase by diethyl *p*-nitrophenyl phosphate has also been shown to reverse readily (Davison, 1953). Thus after inhibition of these enzymes by a series of inhibitors the identity or otherwise of the

inhibited enzyme may be established by an examination of the rate of return of enzyme activity under standard conditions. In this paper experiments will be described using rabbit erythrocyte true cholinesterase with inhibitors containing two methoxy groups and rat serum pseudo-cholinesterase with inhibitors containing two ethoxy groups.

METHODS

The methods for studying reversal of inhibition are those previously described (Aldridge, 1953*a*). The recovery of activity was measured by first treating the enzyme with inhibitor, then removing excess inhibitor by means of enzymes which will hydrolyse it rapidly and finally testing the activity of the inhibited enzyme after the addition of substrate. Rabbit serum will hydrolyse dimethyl and diethyl *p*-nitrophenyl phosphates (Aldridge, 1953*b*) dimethyl and diethyl phosphorofluoridates (Mazur, 1946), and *OO*-dimethyl and *OO*-diethyl-*S*-*p*-nitrophenyl phosphorothiolates. Tetramethyl and tetraethyl pyrophosphates are hydrolysed by erythrocytes (Aldridge & Davison, 1952*a*), and rat erythrocytes were added for this purpose in studying the reversal of inhibition of rat pseudo-cholinesterase.

The enzyme activity was determined using 0·015 M acetylcholine as substrate for the true cholinesterase of rabbit erythrocytes and 0·03 M *n*-butyrylcholine perchlorate for the pseudo-cholinesterase of rat serum. The buffer used contained 0·0357 M-$NaHCO_3$ and 0·164 M-NaCl. All inhibitors were dissolved freshly in buffer for each experiment. Dimethyl and diethyl phosphorofluoridates were synthesized from the corresponding phosphorochloridates by the method of Saunders & Stacey (1948), and tetramethyl pyrophosphate by the method of Toy (1949).

It should be noted that with inhibitions which are reversible, the inhibitory reaction is stopped upon the addition of substrate and the enzyme activity is returning throughout its determination. Although under these circumstances the output of CO_2 is not linear with respect to time, the best straight line through these points (Aldridge, Berry & Davies, 1949) has been calculated and the slope used as a measure of the activity over the period of determination.

RESULTS

The return of true cholinesterase activity of rabbit erythrocytes after dimethyl phosphorus compounds. In Fig. 1 are shown the four inhibitors which have been examined. They are all potent inhibitors of cholinesterase. According to the phosphorylation theory the first three compounds should all produce a dimethyl phosphorylated enzyme. The compound containing a *P*-*S*-*p*-nitrophenol grouping will only do so if the bond breaks between the phosphorus and sulphur atoms. The results shown in Fig. 2 show that the enzyme activity after inhibition by all four compounds returns at the same rate. The half-life for the inhibited enzyme at 37° and pH 7·8 is about 85–90 min.

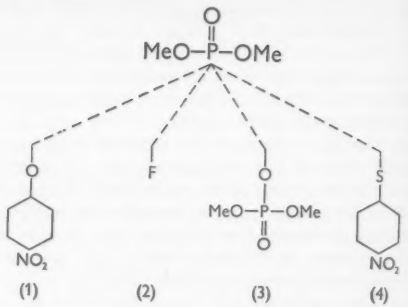


Fig. 1. Inhibitors used containing two methoxy groups. (1) dimethyl *p*-nitrophenyl phosphate. (2) dimethyl phosphorofluoridate. (3) Tetramethyl pyrophosphate. (4) *OO*-dimethyl *S*-*p*-nitrophenyl phosphorothiolate.

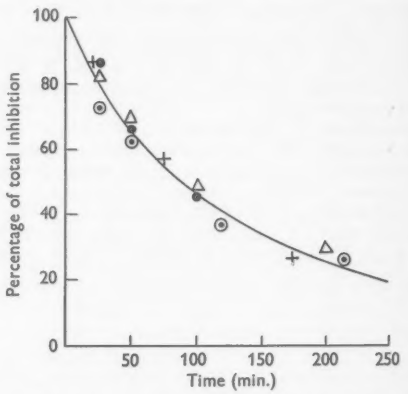


Fig. 2. The rate of return of enzyme activity after the treatment of cholinesterase with inhibitors containing two methoxy groups. After treatment of a suspension of rabbit erythrocytes (15 ml.) with inhibitor for 10 min. at 37°, the preparation was incubated with *A*-esterase (3 ml.) for 15 min. at 37°. Portions were taken and at various times acetylcholine tipped in to determine enzyme activity. The following concentrations of inhibitors were used: +, dimethyl *p*-nitrophenyl phosphate, $2·6 \times 10^{-7}$ M; ●, tetramethyl pyrophosphate, $1·4 \times 10^{-6}$ M; ⊙, dimethyl phosphorofluoridate, $1·1 \times 10^{-6}$ M; △, *OO*-dimethyl *S*-*p*-nitrophenyl phosphorothiolate, $3·6 \times 10^{-7}$ M.

The return of pseudo-cholinesterase activity of rat serum after diethyl phosphorus compounds. The parallel experiments have been carried out using the corresponding diethyl inhibitors and pseudo-cholinesterase of rat serum. As Fig. 3 shows, the rate of return of enzyme activity is the same for all four compounds. The half-life of this inhibited enzyme is approximately 450 min.

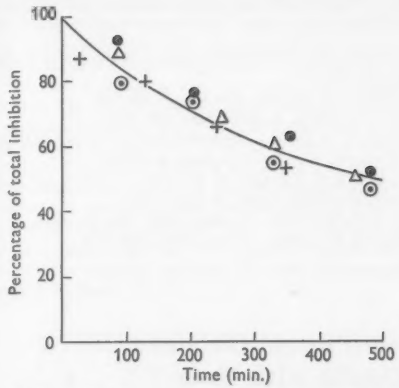


Fig. 3. The rate of return of enzyme activity after treatment of cholinesterase by inhibitors containing two ethoxy groups. After treatment of rat serum (4 ml.) with inhibitor for 30 min. at 37°, the preparation was incubated with A-esterase (1 ml.) for 30 min. at 37°. Portions were taken and at various times butyryl choline tipped in to determine enzyme activity. The following concentrations of inhibitors were used; +, diethyl *p*-nitrophenyl phosphate, 1×10^{-6} M; ●, tetraethyl pyrophosphate, 1×10^{-6} M; △, diethyl phosphorofluoridate, 3×10^{-6} M; ⊙, *OO*-diethyl *S*-*p*-nitrophenyl phosphorothiolate, 1×10^{-6} M.

DISCUSSION

Our evidence for the identity of the inhibited enzymes is based on determinations of the rate of return of enzyme activity under standard conditions. On this basis the results described show that, amongst inhibitors of the general formula $(R)_2(X)PO$ (X is the group labile to hydrolysis), if X is varied and R maintained constant the same inhibited enzyme results. This conclusion has been reached with true cholinesterase of rabbit erythrocytes inhibited by dimethyl phosphorus compounds and also for pseudo-cholinesterase of rat serum using diethyl phosphorus compounds. This is a direct confirmation of the theory of the mechanism of inhibition previously put forward (Aldridge, 1953*a*).

Since the inhibitors containing *P*-*S*-*p*-nitrophenyl groups are more unstable to hydrolysis than the corresponding compounds with *P*-*O*-*p*-nitrophenyl groups (Topley, 1950; Aldridge & Davison, 1952*b*) it would be expected that the corresponding enzyme phosphate would have different stabilities if the phosphorus atom were linked to the enzyme through a sulphur or oxygen atom, respectively. For these inhibitors this is not so. A direct attachment of the phosphorus atom to some atom of the enzyme protein would explain this observation, i.e.

$$E + \underset{\displaystyle SR'}{\overset{\displaystyle (RO)_2}{\overset{|}{P}}}=O \rightarrow E - \underset{}{\overset{\displaystyle (RO)_2}{\overset{|}{P}}}=O + R^1SH.$$

When carboxylic esters are hydrolysed by base catalysis, the C—O—R bond is broken between the carbon and oxygen (Remick, 1949). It has also been shown that for a similar hydrolysis of trimethylphosphate, the bond breaks between phosphorus and oxygen atoms (Blumenthal & Herbert, 1945). Since *p*-nitrothiophenol has a distinctly different colour from *p*-nitrophenol it is easy to show that, when compounds containing *P*-*S*-*p*-nitrophenyl groups are hydrolysed at pH above 7, *p*-nitrothiophenol is liberated. In confirmation the colour slowly disappears upon standing (oxidation) owing to the formation of the insoluble disulphide. A-esterase (Aldridge, 1953*b*) also breaks the bond between P and S. It is clear therefore that during base catalysis, enzymic hydrolysis by A-esterase and inhibition of the true and pseudo-cholinesterase the labile ester bond is broken next to the phosphorus atom. The mechanism put forward by Bergmann, Wilson & Nachmansohn (1950) for the hydrolysis of acetylcholine by electric-eel cholinesterase also involves a break next to the electrophilic group. It would appear, therefore, that the ester bonds break in the same place for both enzymic and non-enzymic hydrolysis.

SUMMARY

1. Reaction of organophosphorus inhibitors (of the general formula $(R)_2(X)PO$ where X is the group labile to hydrolysis) with cholinesterase produces the same inhibited enzyme provided the R groups are maintained constant even though X is varied.

2. This is a direct confirmation of the phosphorylation mechanism for inhibition.

We are grateful to Dr J. A. Cohen (Leiden, Holland) for the specimen of dimethyl phosphorochloridate, to Dr H. Coates (Albright and Wilson Ltd.) for the trimethyl phosphate, *OO*-diethyl *O*-*p*-nitrophenyl phosphate, *OO*-diethyl *S*-*p*-nitrophenyl phosphorothiolate and tetraethyl pyrophosphate and to Dr R. A. E. Galley for obtaining the *OO*-dimethyl *S*-*p*-nitrophenyl phosphorothiolate. Our thanks are also due to Miss J. E. Cremer and Miss J. Wheatley for valuable technical assistance.

REFERENCES

Aldridge, W. N. (1953*a*). *Biochem. J.* **54**, 442.
Aldridge, W. N. (1953*b*). *Biochem. J.* **53**, 117.
Aldridge, W. N., Berry, W. K. & Davies, D. R. (1949). *Nature, Lond.*, **164**, 925.
Aldridge, W. N. & Davison, A. N. (1952*a*). *Biochem. J.* **51**, 62.
Aldridge, W. N. & Davison, A. N. (1952*b*). *Biochem. J.* **52**, 663.
Bergmann, F., Wilson, I. B. & Nachmansohn, D. (1950). *J. biol. Chem.* **186**, 78.
Blumenthal, E. & Herbert, J. B. M. (1945). *Trans. Faraday Soc.* **41**, 611.
Davison, A. N. (1953). *Biochem. J.* **54**, 583.
Mazur, A. (1946). *J. biol. Chem.* **164**, 271.
Remick, A. E. (1949). *Electronic Interpretations of Organic Chemistry*. London: Chapman and Hall.
Saunders, B. C. & Stacey, G. T. (1948). *J. chem. Soc.* p. 695.
Topley, B. (1950). *Chem. & Ind.* S859.
Toy, A. D. F. (1949). *J. Amer. chem. Soc.* **71**, 2268.
Wilson, I. M. (1952). *J. biol. Chem.* **199**, 113.

Physical Studies on a Soluble Protein Obtained by the Degradation of Elastin with Urea

By T. J. BOWEN
Department of Biochemistry, The University, Leeds, 2

(*Received* 27 *April* 1953)

By the treatment of elastin with oxalic acid at 100°, Adair, Davis & Partridge (1951) obtained a soluble protein which, on gentle warming in pH 4–6 buffer solution, turned milky in appearance; cooling reversed the phenomenon. The effect was due to the separation of a second phase consisting of minute drops of protein solution at a different concentration. Electrophoresis failed to show the existence of more than one component except at an ionic strength of 0·02 and pH 3–5 when there was partial separation of two components. Porous dialysis separated the protein into two components of molecular weight (by osmometry) 6000 and 84 000. Only the component of higher molecular weight showed the reversible separation of another phase on warming. Both fractionated and unfractionated material showed only one boundary on sedimentation in the ultracentrifuge, the sedimentation constant being the same in each case.

Hall (1951) showed that elastin could also be brought into solution in boiling 40 % (w/v) urea. The protein obtained after removing the urea by dialysis showed great similarity to that obtained by oxalic acid degradation. This protein has been studied in the work described here. Evidence is presented which can be interpreted in terms of a reversible dissociation of the protein. The dissociation is favoured by dilution.

METHODS AND RESULTS

Preparation of the protein

Bovine ligamentum nuchae was stripped of extraneous material as thoroughly as possible by mechanical means, then finely minced. Approx. 500 g. of the minced material was mixed with 1·5 l. of 1 % aqueous acetic acid and boiled under reflux for 2–3 hr. to convert collagen into gelatin. The mixture was filtered through cloth, squeezed as dry as possible and then weighed approx. 300 g. In order to prepare the urea breakdown product, about 100 g. of this elastin was refluxed with 5½ l. of 40 % (w/v) aqueous urea until a clear solution was obtained. This usually took about 40 hr. The mixture was filtered through cloth and the filtrate dialysed in cellophan for 4 days against running tap water to remove urea, amino acids, etc. The solution was next evaporated under reduced pressure to 2 l. The concentrated solution was brought to pH 10 with NaOH and any residue spun off. HCl was then added to bring the solution to pH 2, any residue being spun off and rejected. The calculated quantity of solid NaCl was added to make the solution 2·5 M, when the protein separated out as a dense opalescent suspension which was centrifuged. The protein was redissolved most easily, giving a pale-yellow solution, in water brought to pH 10 with NaOH. The material obtained resembled that obtained by treating elastin with oxalic acid (Adair *et al.* 1951) in showing the reversible separation of another phase on heating. The temperature at which the milky appearance due to the separation of fine droplets of the second phase appears depends chiefly on the pH. The precise nature of the buffer present is of much less importance than the pH and the concentration of the material. At pH 2–3 an approx. 1 % (w/v) solution shows the phenomenon when merely warmed in the hand, but the higher the pH of the solution the higher is the temperature required. The material gave a negative result when tested for carbohydrate by Molisch's test. It gave an intense biuret reaction.

Physical properties

Sedimentation in the ultracentrifuge. The experiments were carried out with the Spinco electrically driven ultracentrifuge using the analytical cell with 12 mm. channel. Observations were made on solutions containing 0·259, 0·106, and 0·042 % N (w/v). Estimation of concentration was by

micro-Kjeldahl determinations, but biuret colours were used as a secondary method for checking the dilutions of stock protein solutions. All runs were carried out in the buffer of Miller & Golder (1950) at pH 4 and ionic strength 0·2 against which the samples of material had been dialysed for 2 days. The micro-Kjeldahl analyses were carried out on the dialysed material. Rotor speeds giving gravitational fields of approx. 250 000 *g* were used (but revolution-counter readings were used for the actual calculation of sedimentation constants). Fig. 1 shows the variation of the sedimentation constant with concentration after correcting values to pure water as solvent at 20°. No separation into more than one component was observed on runs lasting 1–1·5 hr. after reaching top speed. A typical sedimentation diagram is shown in Fig. 2.

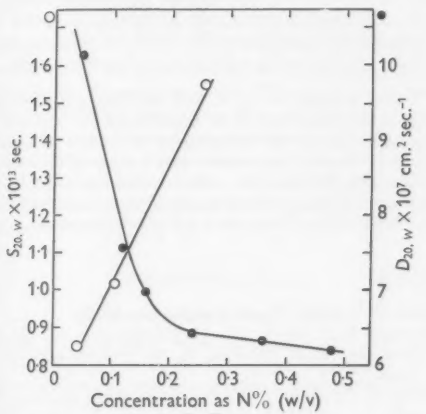


Fig. 1. The variation of the sedimentation constant (○—○) and diffusion coefficient (●—●) with concentration for the elastin degradation product dissolved in buffer at pH 4 and ionic strength 0·2.

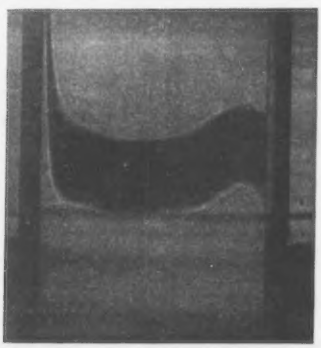


Fig. 2. Sedimentation diagram for protein solution in buffer (pH 4, ionic strength 0·2) after 1·5 hr. at approx. 250000 *g*. Solution contains 0·042% (w/v) N. Direction of sedimentation is from right to left.

Diffusion. A diffusion cell was used similar to that described by Claesson (1946) and made out of stainless steel. The diagonal schlieren optical system was employed and care taken that the light passing through the cell was parallel. The cell was supported in a rigid water bath standing on a concrete floor. Temperature was controlled at 20°±0·01, the circulating pump being remotely situated from the bath to prevent vibration. Diffusion coefficients were calculated by the area-maximum height method. All runs were in the buffer used for the sedimentation work, and were of 2 days duration. Values of the diffusion coefficient (corrected to water as solvent at 20°) are plotted against concentration in Fig. 1.

Partial specific volume. This was found by weighing successive dilutions of protein of known weight fraction in a pycnometer. Temperature was controlled at 20° when filling the pycnometer. The results were calculated according to Kraemer (1940) and the value 0·778 obtained. If a partial specific volume is calculated from the amino acid analysis given for undegraded elastin by Neumann (1949) it is found to be 0·766.

Ultraviolet spectrum. This was determined for the material dissolved in 0·01 N-HCl using a Unicam SP 500 spectrophotometer and is shown in Fig. 3.

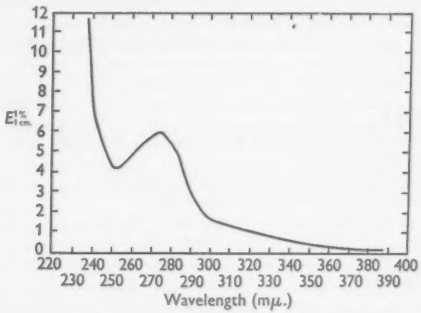


Fig. 3. The ultraviolet-absorption curve in 0·01 N-HCl for the protein obtained by degradation of elastin with urea.

Specific refraction increment. This was calculated from micro-Kjeldahl determinations of concentration and refractive index measurements using an Abbé type refractometer. The value obtained was $\Delta n = 0{\cdot}0118$ for 1% (w/v) of N in the solution.

DISCUSSION

The evidence given above is consistent with the material described being largely polypeptide in nature. The positive variation of the sedimentation constant with concentration, and the converse variation of the diffusion coefficient suggest that the material may exist in solution at pH 4 as a dynamic equilibrium between more than one molecular species, dilution favouring breakdown into smaller molecules. Reversible protein dissociations are well known, e.g. arachin (Johnson, 1946) and insulin (Harfenist & Craig, 1952; Creeth, 1953; Fredericq, 1953). If the rate at which

equilibrium is established between dissociated and undissociated molecules is rapid compared with the rate of separation during sedimentation no resolution of the components is observed and the sedimentation constant assumes an intermediate value.

Adair *et al.* (1951), in their studies on the oxalic acid degradation product of elastin, failed to detect the component of molecular weight 6000 in the ultracentrifuge and obtained identical sedimentation constants for material fractionated by porous dialysis and also for the unfractionated material. This would be explained by assuming an equilibrium between the components.

On extrapolation to zero concentration the sedimentation constant is found to be 0·71 Svedberg unit and the diffusion coefficient $11{\cdot}33 \times 10^{-7}$ cm.^{2} sec.$^{-1}$. These values taken with the value for the partial specific volume lead to a molecular weight at infinite dilution of 6870. In view of the errors involved in the estimation of such a low molecular weight by sedimentation velocity and the nature of the extrapolations this may mean identity with the 6000 molecular-weight component described by Adair *et al.* (1951). A reliable value of the maximum molecular weight approached at high concentrations cannot be given since sedimentation and diffusion coefficients depend on concentration in cases where there is no possibility of dissociation occurring. By analogy with the material prepared by the oxalic acid route a value of 84 000 might be expected for the maximum molecular weight.

SUMMARY

1. The preparation of a soluble protein by treating elastin with hot concentrated aqueous urea is described.

2. Sedimentation, diffusion and other physical measurements have been made on this material.

3. The results have been explained in terms of a reversible dissociation favoured by dilution and a value given for the minimum molecular weight.

I wish to thank Dr D. A. Hall for helpful discussions, Prof. R. D. Passey and Dr L. Dmochowski for ultracentrifuge facilities in the Department of Cancer Research, Mr R. B. Beechey for collaboration in some of the experiments, Mrs M. Lees for technical assistance, Dr R. A. Kekwick and Dr S. M. Partridge for valuable correspondence, and Messrs Firth-Vickers for a gift of stainless steel.

REFERENCES

Adair, G. S., Davis, H. F. & Partridge, S. M. (1951). *Nature, Lond.*, **167**, 605.
Claesson, S. (1946). *Nature, Lond.*, **158**, 134.
Creeth, J. M. (1953). *Biochem. J.* **53**, 41.
Fredericq, E. (1953). *Nature, Lond.*, **171**, 570.
Hall, D. A. (1951). *Nature, Lond.*, **168**, 513.
Harfenist, E. J. & Craig, L. C. (1952). *J. Amer. chem. Soc.* **74**, 3087.
Johnson, P. (1946). *Trans. Faraday Soc.* **42**, 36.
Kraemer, E. O. (1940). *The Ultracentrifuge*, p. 57. Ed. by Svedberg, T. & Pedersen, K. O. Oxford: University Press.
Miller, G. L. & Golder, R. H. (1950). *Arch. Biochem.* **29**, 420.
Neumann, R. E. (1949). *Arch. Biochem.* **24**, 289.

Glycerylphosphorylethanolamine in Rat Brain

By G. B. ANSELL and JOYCE M. NORMAN
Neuropsychiatric Research Centre, Whitchurch Hospital, Cardiff

(*Received* 20 *May* 1953)

The presence of glycerylphosphorylethanolamine (GPE) in aqueous extracts of pig and rabbit liver has been reported by Campbell & Work (1952). More recently, Walker (1952) in carrying out an investigation of the amino compounds in various animal tissues has shown a small amount of glycerylphosphorylethanolamine to be present in ox brain but was unable to detect it in rat brain. Interest in this compound lies chiefly in its possible role as an intermediate in the synthesis of phosphatidylethanolamine, one of the constituents of the kephalin fraction of the phospholipids.

Hahn & Hevesy (1937) demonstrated by means of experiments with radioactive phosphorus (^{32}P) that there is an uptake of phosphorus by the phospholipids of brain tissue, and it has been further shown by Dawson & Richter (1950) that when due allowance is made for the slow rate of the entry of ^{32}P into the brain this uptake is comparable with that of other tissues. Very little, however, is known of the route by which these compounds are synthesized in the tissues. Popják & Muir (1950) investigated possible precursors of the phospholipids using ^{32}P and calculated that α-glycero-

phosphate was a likely intermediate in phospholipid synthesis. Using a partially purified enzyme preparation from rat liver, Kornberg & Pricer (1952*a*, *b*) have shown that L-α-glycerophosphate can serve as a precursor of phospholipids. Kennedy (1953) has also shown that DL-α-glycerophosphate is incorporated into phospholipids by isolated rat-liver mitochondria.

On the other hand, the presence of ethanolamine *O*-phosphoric acid (EPA) in rat brain tissue was demonstrated by Ansell & Dawson (1951), who further showed on the basis of experiments with ^{32}P that it cannot arise solely from the breakdown of phosphatidylethanolamine, and therefore may be a precursor of this phospholipid.

In this paper the presence of glycerylphosphorylethanolamine in rat brain tissue is reported and evidence of its existence in an uncombined form obtained. Experiments with ^{32}P have been carried out in order to determine the relationship of this compound to ethanolamine phosphoric acid on the one hand, and lipid-bound glycerylphosphorylethanolamine on the other. A preliminary account of this work has already been reported (Ansell & Norman, 1953).

EXPERIMENTAL

Chromatography of brain extracts

Young rats (30–100 g.) were decapitated and the heads immediately frozen in liquid oxygen. The brains were dissected out, crushed and weighed. Extracts were then prepared by one of the following procedures.

(i) *Tungstic acid precipitation.* Tungstic acid extracts of brain were prepared as described previously (Ansell & Dawson, 1951), except that, after heating, the mixture was immediately centrifuged. Extracts were desalted in an apparatus similar to that described by Consden, Gordon & Martin (1947). This method was used in all tracer experiments.

(ii) *Trichloroacetic acid precipitation.* To the crushed and frozen brain was added 5 ml. of 12% (w/v) trichloroacetic acid solution, the mixture shaken vigorously and centrifuged immediately. The supernatant was extracted twice with an equal volume of water-saturated ether and the aqueous phase desalted. This method was used only in preliminary experiments.

(iii) *Ethanol precipitation.* This method was based on that of Awapara (1948). The frozen brain was dispersed with a mechanical steel-bladed homogenizer in a volume of ethanol equal to 4 times the water content of the tissue (taken as 80% of the wet weight). After centrifuging, a portion of the supernatant was shaken with four times its volume of $CHCl_3$ and the emulsion centrifuged. The aqueous layer was removed, washed with a little $CHCl_3$ and desalted. This method was used as a mild extraction procedure to confirm the results of experiments with tungstic acid and trichloroacetic acid.

Chromatography. Samples of the desalted extract equivalent to 0·25 g. of brain were subjected to two-dimensional chromatography on Whatman no. 4 paper treated with basic copper carbonate (Crumpler & Dent, 1949). Phenol (A.R.): water (4:1, w/v) was used as the first solvent and collidine-lutidine (Dent, 1948) as the second. Chromatograms were sprayed with 0·2% ninhydrin in 95% ethanol or (in later experiments) with 0·4% ninhydrin in 90% aqueous *iso*propanol containing 5% (v/v) of collidine (Lewis, 1952).

After 16 hr. at room temperature such chromatograms showed four ninhydrin-reacting compounds. In addition to taurine, EPA and γ-aminobutyric acid, a faint spot appeared in close proximity to the γ-aminobutyric acid and in a position similar to that ascribed to GPE (Campbell & Work, 1952). On some chromatograms these two spots could only be distinguished by the sudden change in the intensity of the ninhydrin colour. In general there was a pink haze on the chromatogram; this may have been due to the copper complexes of α-amino acids of which comparatively large amounts were present.

Confirmation of the presence of glycerylphosphorylethanolamine in rat brain

Although Walker (1952) has reported the presence of free GPE in ox brain he states that it is not found in rat brain and it was therefore thought advisable to confirm the identity of the GPE spot as follows.

The presence of an α-aminocarboxylic acid grouping was excluded by the fact that the compound survived the copper treatment. Phosphorus determinations on the paper containing the spot showed that it contained a significantly greater amount of phosphorus than the surrounding paper.

When a rat was injected intracisternally with ^{32}P and a brain extract was prepared in the usual way, the ninhydrin-reacting area believed to be GPE corresponded exactly with a radioactive spot detected by a radioautograph.

Hydrolysis of a brain extract in 5·5N-HCl for 20 hr. at 100° caused this spot to disappear. In a further experiment the position of GPE was located on a number of heated, unsprayed chromatograms by its fluorescence in ultraviolet light (Woiwod, 1949*a*; Fowden, 1951). These areas were cut out and eluted with water. The eluate was hydrolysed for 20 hr. in 5·5N-HCl at 100° and subjected to two-dimensional chromatography in the presence of basic copper carbonate. No GPE spot was visible but a new faint spot appeared in the position of ethanolamine.

When a solution of GPE, prepared from the ether-soluble phospholipids of brain (see below), was run on a two-dimensional chromatogram with a brain extract the spot due to GPE was indistinguishable from the spot obtained from brain extracts. The same result was obtained with a synthetic sample of L-α-GPE (synthesized by Prof. E. Baer and kindly supplied through Dr R. M. C. Dawson). Similarly, the eluted material from the spot believed to be GPE (located on phenol:collidine chromatograms with ultraviolet light) behaved in the same manner as synthetic GPE on single-dimensional chromatograms using *n*-butanol: acetic acid (Woiwod, 1949*b*), *n*-butanol:ethanol:ammonia (Partridge, 1948) or *n*-butanol:formic acid (Wiggins & Williams, 1952) as developing solvents.

Experiments using radioactive phosphorus (^{32}P)

(*a*) *Experiments in vivo.* Young rats (40–150 g.) were used throughout the investigation. In some experiments 120–300 μc of $KH_2{}^{32}PO_4$ were injected intraperitoneally; in

others, 17–60 μc were injected intracisternally by the method of Jeffers & Griffiths (1942). Although the use of an intracisternal injection has the advantage of producing a high local concentration of ^{32}P within the brain (Bakay, 1951) it was difficult to estimate the exact amount of isotope which had entered the cisterna magna.

(*b*) *Separation of the glycerylphosphorylethanolamine phosphorus and the ethanolamine phosphoric acid phosphorus of brain extracts.* Tungstic acid extracts of the frozen brains were desalted for 10–20 min. as this process removed most of the highly radioactive inorganic and acid-labile phosphorus compounds of the brain acid-soluble phosphorus. After chromatography the GPE spot was cut out and an equivalent area, measured by weight, around the spot taken as a control. The EPA spot was large and diffuse; hence only a portion of this spot was taken. In this instance it was not possible to take a surrounding area as a control but, as the amount of phosphorus and radioactivity to be estimated was high, it was considered sufficiently accurate to use an equivalent area of paper some distance away. In general chromatograms were run in duplicate.

All spots and control areas were oxidized as previously described (Ansell & Dawson, 1951), and diluted with distilled water for radioactivity and phosphorus determinations.

(*c*) *Separation of acid-liberated glycerylphosphorylethanolamine phosphorus.* The tungstic acid precipitate of brain was washed twice with 5 ml. of water containing 0·2 ml. of tungstic acid solution. It was then mixed with 5 ml. of 12% (w/v) trichloroacetic acid solution (so that the final concentration of trichloroacetic acid was 10%) and allowed to stand overnight (16 hr.) at 4°. After centrifuging, the supernatant was washed twice with an equal volume of ether and a volume equivalent to approximately 250 mg. of tissue was subjected to two-dimensional chromatography in the usual way. The GPE spot obtained in this way was cut out for phosphorus and radioactivity determinations.

(*d*) *Separation of phosphatidylethanolamine phosphorus.* The brain residue obtained in (*c*) was washed thoroughly with trichloroacetic acid solution and water and finally dried with acetone; it was then mixed with a little sand and allowed to stand in a vacuum desiccator overnight.

The powdered, dried brain residue was extracted for 45 min. with 30 ml. of a boiling 1:1 (v/v) $CHCl_3$:ethanol mixture, the extract taken to dryness under reduced pressure and this lipid residue extracted with 15 ml. of warm ether. After allowing the ether solution to stand at 4° for at least 30 min., it was centrifuged to remove ether-insoluble material. The ethereal solution was taken to dryness under reduced pressure. The residue, containing the ether-soluble phospholipids, was hydrolysed with 2 ml. of 0·08 M-$HgCl_2$ solution on a boiling-water bath for 3 hr. (Norman & Dawson, 1953), to liberate GPE from phosphatidylethanolamine. The GPE was isolated by single-dimensional paper chromatography using phenol as the solvent and the ninhydrin-reacting spot oxidized and diluted in the usual way.

(*e*) *Inorganic plus acid-labile phosphorus.* A sample (0·5 ml.) of the tungstic acid brain extract was used for determination of the specific activity of inorganic + acid-labile phosphorus as previously described (Ansell & Dawson, 1951), except that the radioactivity determination was carried out on the aqueous solution before treatment with molybdate.

(*f*) *Radioactivity and phosphorus determinations.* These were carried out as previously described (Ansell & Dawson, 1951). The radioactivity determinations were subject to a variation of $\pm 2\%$ and phosphorus determinations to a variation of $\pm 2{\cdot}5\%$.

(*g*) *Experiments in vitro.* Minced rat brain was suspended in Krebs bicarbonate-Ringer solution (200 mg. wet weight brain tissue/ml.). This suspension (2 ml.) was incubated with 15 μc. of $KH_2{}^{32}PO_4$ in Warburg vessels for 2 hr. (see Table 3). After incubation the contents of each vessel were treated with 0·22 ml. of tungstic acid and heated for 2 min. at 100°. In order to obtain a sufficient volume of extract the contents of two similarly treated vessels were combined.

For isolation of the GPE in the extracts an amount of extract equivalent to 250 mg. of brain was subjected to two-dimensional chromatography as previously described. A further sample of the extract was then treated with saturated $Ca(OH)_2$ solution until the pH was 8·5. After allowing the calcium phosphate to precipitate at 4° for 15 min., it was removed by centrifugation and the solution treated with Na_2CO_3 solution until no further $CaCO_3$ was precipitated. The supernatant from this was then desalted and an amount equivalent to 50 mg. of brain subjected to two-dimensional chromatography for the isolation of EPA. The calcium precipitation removed most of the inorganic phosphate of high specific activity which might have contaminated the EPA spot because of the proximity of this substance to the origin on the chromatograms.

The brain precipitate was treated as in the *in vivo* experiments to determine the specific activity of the phosphatidylethanolamine.

RESULTS

Evidence for free glycerylphosphorylethanolamine in rat brain. The spot due to glycerylphosphorylethanolamine was seen on chromatograms of brain extracts which were prepared in three different ways from brain tissue frozen in liquid oxygen. Both the tungstic acid and especially the ethanolic precipitation of the proteins are procedures unlikely to cause hydrolysis of glycerylphosphorylethanolamine-containing phospholipids and it seems most likely therefore that a small amount of free glycerylphosphorylethanolamine is present in rat brain tissue. The amount of rat brain extract examined by Walker (1952) would have been too small to demonstrate its presence; the fact that he observed it in comparatively high concentration in cow brain is not completely conclusive because of the inevitable delay in extracting such slaughterhouse material. Three methods of extraction were investigated because it was found that mild treatment with acid, i.e. 10% trichloroacetic acid solution at 4° for 16 hr., released glycerylphosphorylethanolamine from brain tissue. It was also released under these conditions from DL-α-dimyristylkephalin, the amount corresponding to a 1% breakdown of the kephalin.

The strongest evidence for the existence of free glycerylphosphorylethanolamine in rat brain, however, comes from experiments with isotopes. As can be seen in Table 1, the specific activity of the

glycerylphosphorylethanolamine in brain extracts after short exchange periods was always greater than that of the phosphatidylethanolamine and, as will be seen from later discussion, also greater than the plasmalogen (acetalphospholipid). Furthermore, after a 24 hr. exchange, the specific activities of the glycerylphosphorylethanolamine and ethanolamine phosphoric acid in the extracts were identical. This indicates that the glycerylphosphorylethanolamine found in brain extracts is a genuine component of the acid-soluble phosphorus fraction because all acid-soluble phosphorus compounds of nervous tissue are in isotopic equilibrium within 24 hr. (Samuels, Boyarsky, Gerard, Libet & Brust, 1951).

The concentration of free glycerylphosphorylethanolamine in rat brain is very low, the mean value obtained from the figures from twelve young rats being 2·3 mg./100 g. This value must, however, be regarded as a minimum because recovery of the synthetic substance from two-dimensional chromatograms was never greater than 80 %. Nevertheless, it represents a very small fraction of the acid-soluble phosphorus and free amino nitrogen in rat brain extracts.

The uptake of ^{32}P *into the glycerylphosphorylethanolamine of rat brain* in vivo. In experiments on the uptake of ^{32}P into the glycerylphosphorylethanolamine and other phosphorus-containing fractions of brain, intracisternal and intraperitoneal injections of the isotope were used. Although the glycerylphosphorylethanolamine isolated from the brains of animals injected intracisternally had a high rate of counting after short exchange periods, this mode of injection often proved difficult and sometimes failed completely. However, the results were useful in confirming some of those obtained after intraperitoneal injection in which the counting rate of the glycerylphosphorylethanolamine was often very low.

In Table 1 the specific activities of the ethanolamine phosphoric acid, free glycerylphosphorylethanolamine, acid-liberated glycerylphosphorylethanolamine and phosphatidylethanolamine at given times after either intracisternal or intraperitoneal injection of ^{32}P are shown. It may be seen that the specific activity of the free glycerylphosphorylethanolamine 105 min. after intraperitoneal injection was only 10 % of that of the ethanolamine phosphoric acid; after 349 min. this figure had risen to 20 % and after 24 hr. the two compounds were equally radioactive. It can also be seen that the specific activity of the free glycerylphosphorylethanolamine was always higher than that of the phosphatidylethanolamine and hence, after short exchange periods, is intermediate between that of the ethanolamine phosphoric acid and phosphatidylethanolamine.

Table 1. *Specific activities of the inorganic and acid-labile phosphate, ethanolamine phosphoric acid (EPA), glycerylphosphorylethanolamine (GPE), acid-liberated GPE and phosphatidylethanolamine in rat brain after injection of* ^{32}P

Wt. of rat (g.)	Dose ^{32}P (μc)	Exchange period (min.)	Specific activity (counts/min./mg. P)				Ratio of specific activities		
			Inorganic + acid-labile P	EPA	GPE	Acid-liberated GPE	Phosphatidylethanolamine	GPE/EPA	GPE/phosphatidylethanolamine	Acid-liberated GPE/phosphatidylethanolamine
					Intracisternal					
112	60	66	—	Approx. 500 000	11 500	4 530	4 440	0·02	2·5	1·02
80	55	230	—	607 000	154 000	21 050	21 650	0·25	7·1	0·97
150	55	319	—	578 000	90 000	18 950	22 550	0·16	4·0	0·84
					Intraperitoneal					
40	7·5/g.	105	86 200	25 700	<2 400	—	771	<0·09	<3·1	—
39	7·2/g.	240	87 400	51 550	8 000	—	2 740	0·16	2·9	—
45	7·35/g.	349	69 200	56 450	13 000	—	—	0·23	—	—
70	1·7/g.	1420	—	18 500	19 370	<3 600	2 870	1·05	6·8	<1·25
70	1·7/g.	1455	—	20 400	19 800	2 730	2 795	1·03	7·1	0·98
76	5·0/g.	1440	36 800	38 600	33 300	—	—	0·86	—	—

On the other hand, the specific activity of the glycerylphosphorylethanolamine liberated by acid (approximately 10 mg./100 g. brain) was invariably almost identical with that of the phosphatidyl ethanolamine. This indicates either that part of this phospholipid is hydrolysed by trichloroacetic acid, as was found for synthetic DL-α-dimyristylkephalin, or that another fraction with the same turnover rate is acid-labile.

In order to investigate the nature of the acid-liberated glycerylphosphorylethanolamine the specific activity of the glycerylphosphorylethanolamine liberated from the acetalphospholipid fraction of rat brain was determined. From half the brain the total lipid was extracted, saponified and allowed to stand in 10% trichloroacetic acid solution for 2 hr. at room temperature, according to the method of Schmidt, Benotti, Hershmann & Thannhauser (1946). From the acid extract glycerylphosphorylethanolamine was separated by chromatography and the specific activity determined in the usual way; this must have derived from an alkali-stable, acid-labile, glycerylphosphorylethanolamine-containing phospholipid, e.g. acetalphospholipid. The other half of the brain was treated with tungstic acid and then allowed to stand at 4° with 10% trichloroacetic acid solution in the usual way for the determination of the specific activity of the acid-liberated glycerylphosphorylethanolamine. The specific activity of the phosphatidylethanolamine was determined, using the trichloroacetic acid-precipitated brain as previously described.

In Table 2 the specific activities of the acid-liberated glycerylphosphorylethanolamine, phosphatidylethanolamine and alkali-stable, glycerylphosphorylethanolamine-containing phospholipid are shown. It can be seen that the specific activities of all three are very similar.

The amount of glycerylphosphorylethanolamine liberated from the alkali-stable phospholipid was approximately 120 mg./100 g. brain (a minimum value), a considerably greater amount than that liberated from whole brain tissue.

The uptake of ^{32}P *into the glycerylphosphorylethanolamine of rat brain* in vitro. When minced rat brain was incubated for 1–2 hr. in glucose-bicarbonate Ringer solution containing $KH_2{}^{32}PO_4$, there was a small but definite uptake into the glycerylphosphorylethanolamine isolated on chromatograms (Table 3). Its specific activity was very much lower than that of the ethanolamine phosphoric acid but the synthesis of both was increased when the incubations were carried out in the presence of ethanolamine. The radioactivity detected in the phosphatidylethanolamine was not significant, which confirms earlier observations by Dawson (1953).

Table 2. *Specific activities of acid-liberated glycerylphosphorylethanolamine (GPE), phosphatidylethanolamine and alkali-stable, GPE-containing phospholipid of rat brain after the intraperitoneal injection of* ^{32}P

Wt. of rat (g.)	Dose ^{32}P (μc/g.)	Exchange period (min.)	Specific activity (counts/min./mg. P)			Ratio of specific activities	
			Acid-liberated GPE	Phosphatidylethanolamine	Alkali-stable, GPE-containing phospholipid	Acid-liberated GPE/phosphatidylethanolamine	Alkali-stable, GPE-containing phospholipid/phosphatidylethanolamine
64	4·7	119	540	628	622	0·86	0·99
58	5·1	298	1150	1102	1453	1·04	1·32
60	5·0	1484	7160	6180	7460	1·16	1·21

Table 3. *Synthesis of radioactive glycerylphosphorylethanolamine (GPE) by minced rat brain*

(Each Warburg vessel contained 400 mg. minced rat brain, 3·6 ml. Krebs bicarbonate-Ringer solution, 15 μc. $KH_2{}^{32}PO_4$ (3 μg. P) in 0·2 ml., 0·2 ml. of 5·4% (w/v) glucose. Temp. 37°. Gas phase, 95% O_2/5% CO_2.)

	Time of incubation (min.)	Additional substrate	Specific activity (counts/min./mg. P)			
			Inorganic + acid-labile P	EPA	GPE	Phosphatidylethanolamine
Expt. 1	0	—	7640×10^3	0	0	<207
	120	—	6620×10^3	71 000	573	<600
	120	Ethanolamine, 0·04 M	6720×10^3	175 000	2 100	0
Expt. 2	0	—	—	0	0	<226
	60	—	—	—	2 200	<228
	60	Ethanolamine, 0·04 M	—	—	13 500	<455
	120	—	—	—	13 500	<183
	120	Ethanolamine, 0·04 M	—	—	52 200	<226

DISCUSSION

From evidence presented in this paper it appears certain that glycerylphosphorylethanolamine is present in the acid-soluble phosphorus fraction of rat-brain tissue. Because of our incomplete knowledge of the lability of kephalin fractions the fact that it is present in brain extracts prepared from frozen brains under mild conditions is not in itself conclusive. It was demonstrated, for example, that glycerylphosphorylethanolamine is liberated from brain tissue under acid conditions at a low temperature. However, isotopic experiments, both *in vivo* and *in vitro*, demonstrated that its specific activity was not consistent with its being derived from any glycerylphosphorylethanolamine-containing phospholipid, unless it is assumed that the turnover of such phospholipids is extremely heterogeneous.

When ^{32}P was injected intraperitoneally and intracisternally into young rats the specific activity of the glycerylphosphorylethanolamine in the brain extracts after short periods was always greater than that of the phosphatidylethanolamine and therefore glycerylphosphorylethanolamine must be considered as a possible phospholipid precursor. Previously, Ansell & Dawson (1951) suggested that ethanolamine phosphoric acid could not be excluded as a precursor of phosphatidylethanolamine on the basis of a comparison of the specific activities of the ethanolamine phosphoric acid and ether-soluble phospholipids. The direct comparison of the specific activity of ethanolamine phosphoric acid with that of phosphatidylethanolamine reinforces this conclusion. On the other hand, Popják & Muir (1950) are of the opinion that the *in vivo* synthesis of phospholipids, by which they mean phosphatidylethanolamine and phosphatidylcholine, proceeds by way of a common precursor, namely α-glycerophosphate. The results of Kornberg & Pricer (1952*a*) suggest, however, that L-α-glycerophosphate is not an exclusive *in vitro* precursor of phospholipids because they have shown that phosphorylcholine can be incorporated as a unit. In view of these results it would appear that there are alternative pathways for phospholipid synthesis in tissues. It seems unlikely, however, that glycerylphosphorylethanolamine lies on the major synthetic pathway of phosphatidylethanolamine because its specific activity would have to be very much greater in relation to the phosphatidylethanolamine than the experimental finding to compensate for the relatively small amount present in rat brain.

It has been shown by Schmidt *et al.* (1946) that acetalphospholipids are completely broken down in 10% trichloroacetic acid in 2 hr. at room temperature. It has further been demonstrated by Thannhauser, Boncoddo & Schmidt (1951) that the ethanolamine-containing acetalphospholipid yields glycerylphosphorylethanolamine on mild hydrolysis with acid. It would be expected therefore that the acid-liberated glycerylphosphorylethanolamine in the present work was derived from such an acetalphospholipid. This acid-liberated glycerylphosphorylethanolamine (10 mg./100 g. brain) corresponds to 20 mg. acetalphospholipid/100 g. brain (derived from palmitaldehyde) but the amount of acetalphospholipid present in rat-brain tissue calculated from the aldehyde value of Anchel & Waelsch (1944) is 600 mg./100 g.

In an attempt to reconcile these results, experiments were performed in which the glycerylphosphorylethanolamine liberated by acid from the alkali-stable phospholipids was determined. The value obtained corresponded approximately to 240 mg. acetalphospholipid/100 g. brain; this is minimal in view of the incomplete recovery of glycerylphosphorylethanolamine from chromatograms. However, this value for the acetalphospholipid content of rat brain is still considerably lower than that obtained by Anchel & Waelsch (1944) and this may be accounted for by the existence in brain of acetalphospholipids that do not contain ethanolamine (Klenk & Böhm, 1951; Lovern, 1952). The large amount of glycerylphosphorylethanolamine liberated by acid from the extracted acetalphospholipids as compared with that liberated from whole brain tissue would indicate that the lability of acetalphospholipids to acid is very much less in the latter case. It is possible, therefore, that brain acetalphospholipid exists in a combined form within the tissue.*

The fact that synthetic DL-α-dimyristylkephalin is hydrolysed by 10% trichloroacetic acid at 4° suggests that the acid-liberated glycerylphosphorylethanolamine derived partly from ethanolamine-containing acetalphospholipid and partly from phosphatidylethanolamine. If this is so then the two phospholipids must have similar rates of synthesis. This conclusion is supported by the fact that the ethanolamine-containing acetalphospholipid was shown to have a similar specific activity to both the acid-liberated glycerylphosphorylethanolamine and phosphatidylethanolamine.

SUMMARY

1. The presence of a low concentration of free glycerylphosphorylethanolamine (2·3 mg./100 g. brain) in rat-brain extracts has been demonstrated; this concentration is minimal.

* Since this paper was accepted for publication, a report which is in agreement with the above suggestion has come to our notice (Schmidt *et al.* (1953). *Fed. Proc.* **12**, 265) of the comparative stability of the plasmalogens (acetalphospholipids) in an unsaponified, lipid-extracted brain.

2. After injection of ^{32}P into young rats the specific activity of the free glycerylphosphorylethanolamine after short periods was always greater than that of the phosphatidylethanolamine. This suggests that glycerylphosphorylethanolamine is not an *in vivo* breakdown product of phosphatidylethanolamine, although other considerations indicate that it does not lie on the main synthetic pathway.

3. The specific activity of a combined form of glycerylphosphorylethanolamine labile to 10% trichloroacetic acid at 4° was always identical with that of phosphatidylethanolamine. The specific activity of the ethanolamine-containing acetalphospholipid was also similar.

4. The synthesis of radioactive glycerylphosphorylethanolamine by minced rat brain has been accomplished. No *in vitro* synthesis of phosphatidylethanolamine could be demonstrated.

The authors wish to thank the Director of Research, Dr Derek Richter for his continuous interest and encouragement. One of us (J.M.N.) is grateful to the Rockefeller Foundation for a maintenance grant.

REFERENCES

Anchel, M. & Waelsch, H. (1944). *J. biol. Chem.* **152**, 501.
Ansell, G. B. & Dawson, R. M. C. (1951). *Biochem. J.* **50**, 241.
Ansell, G. B. & Norman, J. M. (1953). *Biochem. J.* **54**, viii.
Awapara, J. (1948). *Arch. Biochem.* **19**, 172.
Bakay, L. (1951). *Arch. Neurol. Psychiat., Chicago*, **66**, 419.
Campbell, P. N. & Work, T. S. (1952). *Biochem. J.* **50**, 449.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1947). *Biochem. J.* **41**, 590.
Crumpler, H. R. & Dent, C. E. (1949). *Nature, Lond.*, **164**, 441.
Dawson, R. M. C. (1953). *Biochem. J.* **53**, viii.
Dawson, R. M. C. & Richter, D. (1950). *Proc. Roy. Soc.* B, **137**, 252.
Dent, C. E. (1948). *Biochem. J.* **43**, 169.
Fowden, L. (1951). *Biochem. J.* **48**, 327.
Hahn, L. & Hevesy, G. (1937). *Skand. Arch. Physiol.* **77**, 148.
Jeffers, W. A. & Griffiths, J. Q. (1942). *The Rat in Laboratory Investigation*, eds. Farris, E. J. & Griffith, J. Q. London: Lippincott Co.
Kennedy, E. P. (1953). *J. biol. Chem.* **201**, 399.
Klenk, E. & Böhm, P. (1951). *Biochem. Z.* **288**, 98.
Kornberg, A. & Pricer, W. E. jun. (1952*a*). *Fed. Proc.* **11**, 242.
Kornberg, A. & Pricer, W. E. jun. (1952*b*). *J. Amer. chem. Soc.* **74**, 1617.
Lewis, P. R. (1952). *Biochem. J.* **52**, 330.
Lovern, J. A. (1952). *Biochem. J.* **51**, 464.
Norman, J. M. & Dawson, R. M. C. (1953). *Biochem. J.* **54**, 396.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Popják, G. & Muir, H. (1950). *Biochem. J.* **46**, 103.
Samuels, A. J., Boyarsky, L. L., Gerard, R. W., Libet, B. & Brust, M. (1951). *Amer. J. Physiol.* **164**, 1.
Schmidt, G., Benotti, J., Hershmann, B. & Thannhauser, S. J. (1946). *J. biol. Chem.* **166**, 505.
Thannhauser, S. J., Boncoddo, N. F. & Schmidt, G. (1951). *J. biol. Chem.* **188**, 423.
Walker, D. M. (1952). *Biochem. J.* **52**, 679.
Wiggins, L. F. & Williams, J. H. (1952). *Nature, Lond.*, **170**, 279.
Woiwod, A. J. (1949*a*). *Biochem. J.* **45**, 412.
Woiwod, A. J. (1949*b*). *J. gen. Microbiol.* **3**, 312.

The Bases of the Nucleic Acids of some Bacterial and Animal Viruses: the Occurrence of 5-Hydroxymethylcytosine

By G. R. WYATT
Laboratory of Insect Pathology, Sault Ste Marie, Ontario, Canada

and S. S. COHEN
Department of Pediatrics, Children's Hospital of Philadelphia and Department of Physiological Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania

(*Received* 11 *April* 1953)

Recent studies on the multiplication of viruses have directed attention increasingly toward their nucleic acids. Hershey & Chase (1952) have shown that most, if not all, of the sulphur-containing protein of coliphage T2, which appears to be present in the outer shell of the virus, does not enter the infected cell. However, deoxyribonucleic acid (DNA), apparently organized within the virus, is in some way transferred to the host cell, and appears, therefore, to participate more intimately in the transmission of genetic properties. On infection of *Escherichia coli* with bacteriophage T2, T4 or T6, there is immediate cessation of synthesis of ribonucleic acid (RNA) and net synthesis of DNA is detectable in about 10 min. (Cohen, 1947, 1951). A similar apparent redirection of DNA synthesis during virus multiplication is characteristic of certain induced lysogenic systems, but in this case synthesis of RNA

continues unimpeded (Siminovitch & Rapkine, 1952). Much work has been directed toward tracing the origin of the structural components of phage DNA (e.g. Weed & Cohen, 1951; Putnam, 1952).

It is important, therefore, to know what chemical properties the nucleic acids of bacterial and other viruses may possess. Wide variations have been demonstrated in the composition, with respect to purine and pyrimidine bases, of the RNA's of plant viruses (Markham, 1953) and the DNA's of insect viruses (Wyatt, 1952*b*).

Concerning the DNA of coliphages T2 and T6, conflicting results have appeared. Smith & Wyatt (1951) reported the presence of substantial amounts of cytosine in phage T2; Weed & Cohen (1951) reported isolation of deoxycytidylic acid from T6; Marshak (1951), however, could find only adenine, guanine and thymine in the nucleic acid of T2, and concluded that this virus contained only these three bases. In no case was the total recovery of nitrogenous bases in terms of phosphorus recorded: in the investigation of Weed & Cohen, a total analysis was not the object, and in the other two studies it was assumed that procedures found satisfactory with DNA from other sources would give quantitative results with phage DNA also.

We have re-examined the DNA's of phages T2, T4 and T6, and found that they do contain no cytosine but instead a hitherto unrecognized pyrimidine base, now identified as 5-hydroxymethylcytosine. We report the quantitative purine and pyrimidine composition of the DNA's of these viruses and also qualitative and quantitative results on some other viruses which were examined with the object of determining the distribution of the new base. Preliminary notices from this investigation have already appeared (Wyatt & Cohen, 1952, 1953).

MATERIALS

Phages T2, T4 and T6

The six viruses which provided the basis of these studies were the r and r$^+$ strains of coliphages T2, T4 and T6. Their properties and the isolation of many of the preparations used have been described (Cohen & Arbogast, 1950*b*). Various new preparations were isolated from lysates of high titre (5×10^{11}–10^{12} virus particles/ml.) obtained by growing *Esch. coli*, strain B, to 3×10^9 bacteria/ml. and infecting these cells with an average of five virus particles each. The cells grew exponentially to this level when suspended in 350 ml. of medium at 37° in a 2 l. flask rotated in such a fashion that the medium was thinly layered on its inner surface, thereby providing maximal aeration. After infection, rotation was continued until lysis. The glucose and nitrogen content of the basal mineral medium were fortified tenfold.

In addition, two large preparations of phage T6r$^+$, totalling 35 g., were generously prepared for us by Dr J. Spizizen of the Research Division of Sharp and Dohme, Inc. Because of difficulties attending the purification of large quantities of virus, these preparations contained a certain proportion of non-virus material. They contained about 20% of phage DNA.

For isolation of DNA the phages were disrupted by urea (3·6 g./10 ml. of virus suspension: Cohen, 1947) and deproteinized in M-NaCl-urea solution by $CHCl_3$:*n*-octanol (8:1, v/v). The nucleic acids were precipitated with 4 vol. of cold ethanol and washed in 80%, 90% and absolute ethanol and ether. The fibrous solids were dried *in vacuo* over P_2O_5.

Other viruses

Phage T7. Two large preparations containing *Esch. coli* phage T7 were made available to us by Dr J. Spizizen. They contained about 4% of DNA. From one of these, nucleic acid was isolated by the urea technique. A small preparation (5 mg.) of T7 virus was also provided by Dr L. M. Kozloff of the Department of Biochemistry, University of Chicago. This contained about 10% of DNA.

Phage T5. A preparation of this coliphage, containing about 26% of DNA, was the gift of Dr G. Lark, of New York University School of Medicine. From a portion of it, DNA was isolated by the procedure described by Smith & Wyatt (1951), modified by incubating in N-NaOH at room temperature (20°) instead of 37°. NaOH was used in preference to urea in case traces of RNA might be present. We established, using DNA of phage T6, that 5-hydroxymethylcytosine withstands this treatment without loss.

Polyhedral virus. Polyhedral virus from caterpillars of *Colias philodice eurytheme* Bdvl., for which we are indebted to Dr E. A. Steinhaus of the University of California, was isolated by the method of Bergold (1947, 1953).

Vaccinia virus. This material (160 mg., containing about 2·1% of DNA) was prepared for us by Dr A. L. Brown of the Research Division of Sharp & Dohme Inc., using the method of Hoagland, Smadel & Rivers (1940). Treatment of this virus with urea failed to release nucleic acid. DNA was therefore isolated by the procedure of Smith & Wyatt (1951), incubating in N-NaOH at 20°.

Meningo-pneumonitis virus. A preparation of this virus, a member of the psittacosis group, was the gift of Dr M. M. Sigel, of the Research Department, Children's Hospital of Philadelphia. The virus was isolated by centrifugation from chick allantoic fluid, and we were supplied with 17 mg., containing about 2·5% of DNA.

Pyrimidine derivatives

5-Hydroxymethylcytosine and 5-hydroxymethyluracil were synthesized at our suggestion and kindly made available to us by Dr C. S. Miller, of the Research Division of Sharp and Dohme, Inc. The former was prepared* by reduction of ethyl cytosine-5-carboxylate with $LiAlH_4$, and the latter by the method of Litzinger & Johnson (1936).

A specimen of 5-hydroxyuracil, prepared by the method of Davidson & Baudisch (1925), was the gift of Dr G. H. Hitchings. A specimen of 4-methylcytosine, prepared by Dr Hitchings by a method analogous to that used by Hitchings, Elion, Falco & Russell (1949) for cytosine, was obtained through the courtesy of Dr A. Bendich.

* A description of this synthesis is in preparation by Dr Miller and will be submitted to the *Journal of the American Chemical Society*.

EXPERIMENTAL AND RESULTS

Evidence for the presence of an unknown component in phages T2, T4 and T6

Since hydrolysis with perchloric acid (70% at 100° for 1 hr.: Marshak & Vogel, 1951) had proved satisfactory for quantitative liberation of DNA bases from insect viruses, this method was tried first for analysis of phages T4 and T6. The bases were separated by paper chromatography using an *iso*propanol:water:HCl mixture as the solvent, and eluted and estimated as previously described (Wyatt, 1951*b*, 1952*b*). The principal products were guanine, adenine and thymine, in total amount equivalent to 75–80% of the virus P. The chromatograms also showed weak spots of a substance having an R_F value equal to that of cytosine (0·46–0·50), the yield of which (1–5 moles/100 g. atoms P, using the extinction coefficient of cytosine) varied widely from one experiment to another. On elution, this substance proved to have its ultraviolet-absorption maximum at a slightly longer wavelength than has cytosine, and on being rechromatographed with 86% (v/v) aqueous *n*-butanol as the solvent its R_F (about 0·07) was much smaller than that of cytosine. The substance was largely unchanged by heating with formic acid at 175° for 2 hr. and so was unlikely to be a nucleoside. In addition, its ultraviolet-absorption spectrum exhibited a shift in alkaline solution similar to those of cytosine and 5-methylcytosine, which the ribosides and deoxyribosides of these bases lack.

It was then found that by using formic acid (88% at 175° for 30 min.) for hydrolysis of the viruses the yield of the unknown was much increased (8–12 moles/100 g. atoms P), the total recovered bases now corresponding to 85–90% of virus P. These observations suggested the presence in phage nucleic acid of a relatively labile pyrimidine base different from any previously reported.

A small amount of the substance, obtained by evaporation of the eluate of a chromatogram spot, was deaminated by treatment with HNO_2 (35 μl. of 2M-$NaNO_2$ and 7 μl. of glacial acetic acid were added and, after standing overnight at 27°, the solution was applied directly to paper for chromatography). The product bore a similar relationship, in spectral and chromatographic properties, to uracil as the parent substance did to cytosine.

The structure of the unknown was suggested to us by study of its spectral and chromatographic properties, as already briefly reported (Wyatt & Cohen, 1952, 1953). Ultraviolet-absorption spectra were read at pH 1, pH 7–8 and pH 13 (Fig. 1, Table 1). From among a number of pyrimidine bases which we examined or whose spectra have been published, only cytosine and 5-methylcytosine exhibited shifts of absorption maximum with change of pH closely paralleling those of the unknown. This suggested that our substance had the polar substituents (2-hydroxy-6-amino-) of cytosine and was additionally substituted in the 5-position. Substitution at C-4 (e.g. 4-methylcytosine) diminishes the extent of the spectral shift in alkali. Addition of an ionizable group at C-5 (e.g. 5-hydroxyuracil) changes the spectrum radically, hence the required substituent should be non-ionizable.

The behaviour of the unknown on paper chromatograms in solvents differing as to pH and to water content, as described in our earlier communications, also suggested ionizable groupings identical with those of cytosine and further indicated that the additional substituent should be hydrophilic in nature. As 5-hydroxycytosine had been eliminated, 5-hydroxymethylcytosine seemed a probable structure.

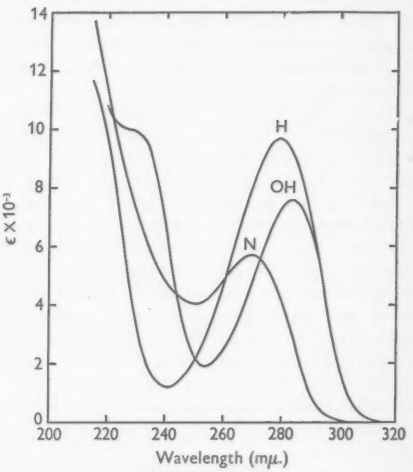


Fig. 1. Ultraviolet-absorption spectra of 5-hydroxymethylcytosine in 0·1N-HCl (H), 0·1N-NaOH (OH) and at pH 7·4 (N). ϵ is the molecular extinction coefficient. See Table 1.

Table 1. *Ultraviolet-absorption data*

(ϵ is the molecular extinction coefficient.)

		Maxima		Minima	
Substance	Solvent	Wavelength (mμ.)	ϵ	Wavelength (mμ.)	ϵ
5-Hydroxymethylcytosine	0·01M Sodium phosphate buffer (pH 7·4)	269·5	5710	251	4060
	0·1N-HCl	279·5	9700	241·5	1230
	0·1N-NaOH	283·5	7590	254	1890
5-Hydroxymethyluracil*	Buffer (pH 7·4) as above	261	—	231	—
	0·1N-HCl	261	—	231	—
	0·1N-NaOH	285	—	245	—

* Measured from eluates of chromatogram spots; the substance was not isolated in crystalline form.

Isolation and characterization of 5-hydroxymethylcytosine from phage T6

To establish with certainty the identity of the supposed new base it was desirable to isolate in pure form a sufficient quantity for elementary analysis and comparison with synthetic material. For this we were fortunate in having the large phage preparations provided by Dr Spizizen. Since the lability of the new base, unique among natural pyrimidines, was not at first fully realized, yields were much lower than they might have been. Nevertheless, a satisfactory sample was isolated by the following procedure.

From 4·5 g. of the virus concentrate, DNA was prepared by the urea technique referred to above. The product weighed 665 mg. and had N/P = 3·85 (atomic proportions).

For removal of purines (Tamm, Hodes & Chargaff, 1952), 500 mg. of T6 DNA were dissolved in 50 ml. of water and dialysed for 24 hr. at 32° against two changes (each 1000 ml.) of 0·03 N-HCl (pH 1·6), followed by dialysis for 3 days against three changes of distilled water at room temperature. The solution from inside the cellophan dialysis sac was concentrated under reduced pressure and freeze-dried. The yield of 'apurinic acid' was 235 mg., from which some 80% of the original purines had been removed without change in the ratio pyrimidines/P. It was evident, however, that some pyrimidine-containing material had passed through the dialysis membrane, and an additional yield corresponding to about 45 mg. was recovered from the last dialysis water.

220 mg. of this 'apurinic acid' were hydrolysed with 1·5 ml. of 88% formic acid at 165° for 20 min. It is clear from later experiments that the yield of hydroxymethylcytosine would have been greatly increased by use of a larger volume of formic acid. The hydrolysate was spread as a band near one edge of a sheet (18·25 × 22·5 in.) of Whatman no. 3 filter paper and chromatographed in the *iso*propanol: HCl solvent. The appropriate band was eluted and the eluate concentrated and chromatographed in a similar way with water-saturated *n*-butanol as the solvent on Whatman no. 3 paper which had been washed with distilled water. The ultraviolet absorption of the eluate from this second chromatogram indicated the presence of some 12 mg. of pyrimidine derivative.

Several fractions obtained in a similar manner, containing altogether about 30 mg. of pyrimidine derivative, were combined and treated with 60 mg. of picric acid. The picrate crystallized in needles. After removal of picric acid (by extraction with toluene and ether in the presence of H_2SO_4, the latter being then removed with $Ba(OH)_2$) the solution was concentrated and the free base crystallized in prisms (21·5 mg.). After two recrystallizations from water the product weighed 15·4 mg. Its elementary composition was compatible with the proposed structure. (Found, after drying *in vacuo* at room temperature: C, 40·2; H, 5·35. $C_5H_7O_2N_3$, 1/2 H_2O requires C, 40·0; H, 5·37%. Loss of weight on drying *in vacuo* at 100° over P_2O_5, 4·7% (required: 6·00%). Found, on exhaustively dried material: N (Dumas), 29·3. $C_5H_7O_2N_3$ requires N, 29·8%.)

At approximately the same time as the natural substance was obtained in pure form, synthetic 5-hydroxymethylcytosine was made available to us by Dr Miller. With respect to their ultraviolet-absorption spectra (Table 1, Fig. 1) and to their movement on paper chromatograms in several solvents, the natural and synthetic substances were identical. On heating, both specimens charred slowly above 200°, without melting up to 300°. 5-Hydroxymethyluracil, subsequently prepared by Dr Miller, was similarly indistinguishable from the deamination product of the natural base. We conclude that the base isolated from bacteriophage T6 is 5-hydroxymethylcytosine.

Quantitative hydrolysis of bacteriophage deoxyribonucleic acid

In view of the failure to obtain good yields of 5-hydroxymethylcytosine from DNA by hydrolysis with $HClO_4$, it was of interest to determine the effect of this acid on the pure base. Accordingly, 0·5 mg. of the synthetic base was heated with 0·05 ml. of 72% $HClO_4$ at 100° for 1 hr. Unexpectedly, 97% of the initial 5-hydroxymethylcytosine was recoverable after heating. However, since the nucleoside and nucleotide of this base are not found after hydrolysis of phage DNA with conc. $HClO_4$ at 100°, the low recovery must result from destruction and not from incomplete liberation. This loss may be due either to weakening of the pyrimidine ring by the glycosidic linkage at N-3 or to the effect of other substances in the hydrolysate, such as H_3PO_4. We have not tested these possibilities experimentally.

For quantitative analysis of the DNA of phages T2, T4 and T6, we hydrolysed with formic acid. When a proportion of formic acid to nucleic acid was used similar to that previously used for DNA from other sources, the yield of hydroxymethylcytosine was somewhat variable, and the total recovered bases were equivalent to only some 90% of the DNA P. When pure hydroxymethylcytosine was subjected to these hydrolytic conditions in the presence of thymus DNA, then separated from the other bases by 2-dimensional chromatography and estimated, only some 70–75% of the added amount was recovered (Table 2, expts. 1 and 2). Alteration of time or temperature of

Table 2. *Recovery of 5-hydroxymethylcytosine subjected to hydrolytic conditions in 88% formic acid at 175° for 30 min. in the presence of thymus deoxyribonucleic acid*

Expt. no.	Formic acid (μl./mg. DNA)	5-Hydroxymethylcytosine (moles/g. atom P) Added	Recovered	Recovery (%)
1	80	0·146	0·099	68
	80*	0·146	0·066	45
2	80	0·175	0·134	77
	80*	0·175	0·108	62
3	260	0·215	0·187	87
4	360	0·206	0·195	95
5	600	0·330	0·318	96·5

* Protein (bovine serum albumin, equal in wt. to the DNA) was also added.

hydrolysis did not lead to significant improvement, but recovery of this base was found to depend markedly on the volume of formic acid used. With a sample of 1·5 mg. of phage, or 0·7 mg. of phage DNA, for which 0·05 ml. of 88% formic acid had been used, maximal yields in terms of P are obtained with 0·25–0·5 ml. By using sufficient formic acid, recovery of hydroxymethylcytosine subjected to hydrolysis along with thymus DNA was raised to approximately 96% (Table 2), and the total base recovery from phage DNA became equivalent to 97–99% of total P.

It has also been found important to avoid an excessive volume of air above the formic acid during hydrolysis. This leads to loss of hydroxymethylcytosine, which can, however, be prevented by replacing the air in the tube with nitrogen or formic acid vapour before sealing it off. Evidently the compound is most stable under reducing conditions, and this may account for the efficacy of formic acid in liberating nucleic acid bases without loss. With sufficient formic acid in a small enough tube, as described below, however, there was no advantage in replacing the air with a non-oxidizing atmosphere.

The increased volume of formic acid was found to lead also to slightly improved yields of other bases, especially guanine, from DNA. When ox-spleen DNA was hydrolysed under these conditions total base recovery was equivalent to 98–100% of the P (Table 6). It appears that the use of an insufficient volume of formic acid has been one reason for low total recoveries of bases from DNA in some previous analyses (e.g. Wyatt, 1951*b*; Chargaff, Lipshitz, Green & Hodes, 1951; Laland, Overend & Webb, 1952).

It will be noted that the formic acid referred to above is 88% (Merck reagent), although in previous work 98–100% (A.R.) has been used. When the two grades were tested simultaneously, no significant difference was found in yields of the bases from DNA. A similar dependence of yield upon volume was found with 98% as with 88% formic acid, and the former produced a darker-coloured hydrolysate suggesting greater degradation of the deoxypentose.

The procedure ultimately adopted for base analysis of phage DNA is as follows. Virus (1·5 mg.) or virus DNA (0·7 mg.) is weighed into a Pyrex glass tube of 6 mm. internal diameter. Formic acid (0·5 ml. of 88%) is added, and the tube is sealed off about 20 mm. above the surface of the liquid and heated to 175° for 30 min. The tube is opened, the hydrolysate evaporated to dryness under reduced pressure at a temperature not exceeding about 75°, and the residue redissolved in 25 μl. of N-HCl. Two 8 μl. portions are taken for chromatography, and two 2 μl. portions for P estimation.

Elementary analyses

P was estimated in the virus and nucleic acid preparations by the method of Bergold & Pister (1948), and N by a micro-Kjeldahl procedure. For the elementary analyses on purified 5-hydroxymethylcytosine we are indebted to the analytical laboratory of the Research Division of Sharp & Dohme, Inc.

Determinations of deoxyribonucleic acid bases in viruses and other materials

Since it was possible that some of the virus preparations might contain RNA, the early chromatograms of phages hydrolysed with $HClO_4$ (which breaks down RNA as well as DNA to free bases) were examined for possible uracil. The ratio of uracil to thymine should give an approximate indication of the ratio of RNA to DNA. Maximal values for uracil, calculated from the absorption at 260 mμ. of the appropriate areas of chromatograms, ranged from 1·9 to 2·8% of the thymine for seven preparations of T4r$^+$, T4r, T6r$^+$ and T6r; in no case did the absorption curve exhibit a peak at this wavelength, so that the absorption must have been largely due to other degradation products from the virus, and any actual uracil was considerably less than the figures mentioned.

The relative proportions of the DNA bases found in the r and r$^+$ mutants of coliphages T2, T4 and T6 are shown in Table 3. The small difference in apparent thymine content of whole viruses compared with isolated DNA has no biological significance, but is due to interference by breakdown products of the non-DNA moiety of the virus. The very small differences in results from the various virus strains are not significant, but fall within the range of experimental error. Because of the lability of 5-hydroxymethylcytosine it is probable that even under the conditions of hydrolysis finally adopted a small amount of this base is lost. The results in Table 2 suggest that this may be of the order of 4%, and the figures presented may require corresponding correction.

From the ratios of total recovered bases to total P (Table 3) it is evident that in the best virus preparations the P may be fully accounted for as nucleotides. The possible presence of 1 or 2% of non-DNA P is not excluded, however,

Table 3. *Composition of phages T2, T4 and T6 with respect to DNA bases, phosphorus and nitrogen*

Material analysed	No. of preparations	No. of analyses	Mean estimated content of bases (moles/100 moles)				Total bases/P (moles/g. atom)	N/P (atomic ratio)
			Adenine	Thymine	Guanine	5-Hydroxymethylcytosine		
T2r$^+$ DNA	2	3	32·5	32·6	18·2	16·7	0·97	—
T2r DNA	1	3	32·4	32·4	18·3	17·0	0·98	—
T6r$^+$ DNA	2	10	32·5	32·5	18·3	16·7	0·99	3·85
T2r$^+$ virus	1	4	32·0	33·3	18·0	16·8	0·99	7·0
T2r virus	1	4	32·3	33·4	17·6	16·7	0·95	7·0
T4r$^+$ virus	1	3	32·3	33·1	18·3	16·3	0·96	6·9
T4r virus	2	3	32·2	33·5	18·0	16·3	0·94	6·7
T6r$^+$ virus	3	2	32·5	33·5	17·8	16·3	0·99	7·2
T6r virus	2	3	32·3	33·4	17·7	16·6	0·88	6·7

because base estimation in the presence of protein may err slightly on the high side. In other preparations some non-DNA P is indicated: this is generally greater in the r than in the r^+ mutants, and in two preparations of T6r averaged some 10% of the total P. This is in general agreement with the results of an earlier study, which indicated that the non-DNA P is associated with contaminants of host origin (Cohen & Arbogast, 1950*a*).

As additional characterization of the viruses, N/P ratios are included in Table 3. These are very similar for the different preparations, tending, as would be expected, to be lower in those containing extraneous P. In spite of some earlier evidence that T4 may contain less DNA per infectious unit than T2 and T6 (Cohen & Arbogast, 1950*a*), no significant difference is evident in the N/P ratios of the three viruses. Our values agree well with the mean value of 6·83 calculated from the data of Herriott & Barlow (1952) for T2.

The discovery of 5-hydroxymethylcytosine in these viruses naturally raised the question whether this substance might occur elsewhere. The biological materials listed in Table 4 were examined. Formic acid hydrolysates were subjected to chromatography in two solvents, as previously described for estimation of 5-methylcytosine (Wyatt, 1951*a*), so that maximal figures for the contents of hydroxymethylcytosine relative to cytosine were obtained. In no case did the eluate of the appropriate region of the chromatogram have the characteristic absorption spectrum of hydroxymethylcytosine, so that no evidence was obtained for the presence of this substance in any of these materials. Where only a small amount of material containing a low proportion of DNA was available, the figures for maximal hydroxymethylcytosine are necessarily raised by interfering substances.

Table 4. *Maximal contents of 5-hydroxymethylcytosine, calculated from the ultraviolet absorption of chromatogram eluates, in various biological materials*

Material examined	Wt. hydrolysed (mg.)	Maximal 5-hydroxymethylcytosine as percentage of cytosine (mol.prop.)
Dried *Esch. coli*, strain B	270	0·2
Crude DNA from *Esch. coli**	—	0·6
Ox-spleen DNA	18	0·2
Phage T5	2·1	1·0
Phage T7†	3·8	2·7
Crude DNA from phage T7‡	18	0·5
Polyhedral virus	10·6	2·4
DNA from vaccinia virus	1·2	0·6
Meningo-pneumonitis virus	14·8	3·9

* Isolated by the method of Smith & Wyatt (1951) from 400 mg. of dried bacteria.
† Preparation of Dr Kozloff.
‡ Isolated from the preparation of Dr Spizizen.

By a similar analysis of phage T6r^+, using 45 mg. of virus, its maximal content of cytosine was limited to 0·2% of its hydroxymethylcytosine.

During the course of this investigation, DNA preparations were obtained from phage T5 and from vaccinia virus of sufficient apparent purity to justify our reporting the molar ratios of the bases found in them. These are shown in Table 5, along with a new analysis of ox-spleen DNA. This was the preparation previously described and analysed (Wyatt, 1951*b*), which is apparently identical in composition with calf-thymus DNA, and the present analysis is considered to be more accurate because of better recoveries resulting from the use of a higher proportion of formic acid in hydrolysis. Quantitative analyses were also performed on phage T7; however, since there was evidence of interference by materials other than DNA, we do not report the figures and merely remark that the results indicated a closer approximation to equimolar proportions of the four bases than are in the DNA of phage T5.

DISCUSSION

It is necessary to reconcile certain earlier results with those now presented. The report of cytosine as a component of phage T2 by Smith & Wyatt (1951) was based on a limited amount of material, and when chromatograms were obtained identical in appearance with those of DNA from other sources it was assumed without further critical examination that spots having equal R_F values represented the same substances. The mistaken recognition of deoxycytidylic acid by Weed & Cohen (1951) resulted from the following coincidence. When the products of hydrolysis of phage T6 DNA in N hydrochloric acid were separated on paper chromatograms, a band was resolved having both the R_F value and the ultraviolet-absorption maximum (278 mμ. in 0·01N hydrochloric acid) of deoxycytidylic acid, which it was therefore assumed to be. Recent re-examination of this fraction by Dr Weed (personal communication), however, has resulted in its resolution on ion-exchange columns into two components: one with absorption maximum at 282 mμ. at pH 2, representing deoxy-5-hydroxymethylcytidylic acid, and the other, with a maxi-

Table 5. *Base composition of DNA's estimated after hydrolysis in 400–700 times their weight of 88% formic acid*

Source of DNA	No. of analyses	Moles/100 moles estimated bases				Total bases/P (moles/g. atom)
		Adenine	Thymine	Guanine	Cytosine	
Ox spleen	4	27·9	27·3	22·7	20·8*	1·00
Phage T5	2	30·3	30·8	19·5	19·5	0·93
Vaccinia virus	1	29·5	29·9	20·6	20·0	1·00

* 1·3 moles of 5-methylcytosine are also present (Wyatt, 1951*b*); this base was not estimated in the present analyses.

mum 274 mμ., as yet unidentified. When combined, these products had an absorption spectrum close to that of deoxycytidylic acid. The conclusions drawn from these studies, however, are not altered by the substitution of hydroxymethylcytosine for cytosine.

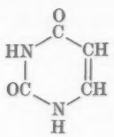
Marshak (1951) missed hydroxymethylcytosine because of his use of perchloric acid for hydrolysis along with a chromatogram solvent system in which it happens to migrate together with guanine. This accounts for the anomalous absorption spectrum for guanine which he reported.

In spite of the considerable evidence that DNA may play a specific role in the transmission of hereditary characters, we were unable to demonstrate any difference in the composition of the DNA of the r and r^+ mutants of phages T2, T4 and T6. This confirms the inference drawn from similar analyses on a number of insect viruses (Wyatt, 1952*b*) that genetic difference is not necessarily accompanied by a detectable quantitative difference in DNA composition.

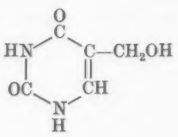
A common pattern has been noted in the composition of DNA from many sources: the molar ratios (adenine)/(thymine) and (guanine)/(cytosine + 5-methylcytosine) are relatively constant and close to unity (Chargaff, 1951; Wyatt, 1952*a*). The same regularities are seen to be valid with DNA from phage T5 and from vaccinia virus, and also with DNA of phages T2, T4 and T6 except that here cytosine is replaced by 5-hydroxymethylcytosine. Whether these near-unity ratios actually signify equal numbers of the corresponding nucleotides in the molecule is as yet uncertain. The present studies, however, have served to emphasize how quantitative errors can result from small differences in experimental conditions and purity of materials, and it is our experience that successive improvements in technique have resulted in bringing the observed ratios successively closer to unity. One is tempted to speculate that regular structural association of nucleotides of adenine with those of thymine and of guanine with those of cytosine (or its derivatives) in the DNA molecule requires that they be equal in number. There is as yet, however, no direct evidence for such a theory.*

The occurrence of 5-hydroxymethylcytosine as a major constituent of the nucleic acid of a virus, none of which could be found in the host cells, presents problems of fundamental importance for the chemistry of virus production. Although discussion must at present remain largely speculative, certain possibilities may be pointed out.

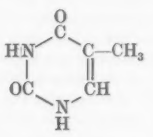
We are concerned with the following pyrimidine bases:



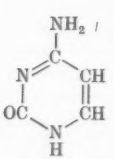
Uracil



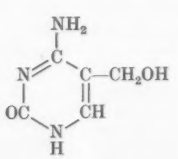
5-Hydroxymethyluracil



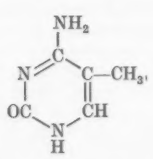
Thymine



Cytosine



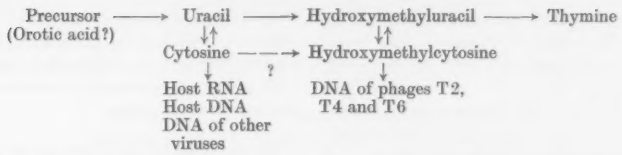
5-Hydroxymethylcytosine



5-Methylcytosine

The metabolic pathways for pyrimidines appear generally to involve their ribosides and deoxyribosides rather than the free bases, and preliminary experiments by one of us (S. S. C.) indicate that this probably is the case in *Esch. coli*. In the rat, Reichard & Estborn (1951) have demonstrated that deoxycytidine can be utilized for production of thymidine, but not vice versa. Elwyn & Sprinson (1950) have implicated the β-carbon of serine as a source of the 5-methyl group of thymine, which is evidently synthesized by methylation of a preformed pyrimidine ring. Since serine cleaves to formaldehyde, we may question whether methyl-group synthesis from serine may not involve an initial hydroxymethylation followed by reduction. If this is so, 5-hydroxymethylpyrimidines (or their deoxyribosides) could be normal metabolites, inter-

* Since this was written; a structure for DNA involving such specific pairing of nucleotides has been proposed by Watson & Crick (1953).



mediary in the synthesis of 5-methylpyrimidines, and a scheme (as above) may be tentatively proposed.

Folic acid has been implicated in the synthesis of the 5-methyl group of thymine (Goldthwait & Bendich, 1952), and it may be with the proposed hydroxymethylation step that it is concerned. This suggests a possible explanation for the inhibition of growth of phages T2, T4 and T6 by sulphanilamide under conditions (including the presence of thymine) which do permit multiplication of phages T1, T3 and T7 (Rutten, Winkler & de Haan, 1950). If the drug interferes with hydroxymethylation, so long as thymine is provided, only the growth of viruses requiring a hydroxymethylpyrimidine will be inhibited. It was with this in mind that we examined the virus of meningo-pneumonitis, which is also sensitive to sulphonamides. It does not contain hydroxymethylcytosine, hence its inhibition must be otherwise accounted for, and may be due to another function of folic acid. The theory with respect to phages T2, T4 and T6 is neither supported nor necessarily invalidated.

The proposal that 5-hydroxymethylcytosine can be derived from cytosine is supported by recent tracer experiments with *Esch. coli* infected with phage T6 (Weed & Cohen, unpublished). Two routes are possible, however: (*a*) the hydroxymethyl substituent may be added directly to cytosine (or its deoxyriboside), or (*b*) cytosine may be deaminated to uracil, which would be hydroxymethylated and then aminated to produce hydroxymethylcytosine. In the latter case 5-hydroxymethyluracil would be a precursor both of thymine and of hydroxymethylcytosine, and the last compound could be an abnormal end product produced only during growth of one of the viruses requiring it.

In either case the problem arises as to whether the virus provides the enzyme for the terminal step in the synthesis of the deoxyriboside of hydroxymethylcytosine or whether the host contains an enzyme which is not normally functioning to produce significant amounts of this compound. Preliminary data suggest that neither the pyrimidine nor its deoxyriboside is a normal intermediate in pyrimidine metabolism (Cohen, unpublished) and the mechanism which permits the new compound to be produced during some types of virus growth is a subject for continued investigation.

To what extent can this special pyrimidine contribute to an explanation of the changes in nucleic acid metabolism observed during virus multiplication? A reorganization of DNA synthesis appears to be a general phenomenon in virus-infected bacteria, and cannot depend on any unusual pyrimidine base. Total inhibition of RNA synthesis, however, has as yet been clearly demonstrated only with phages T2, T4 and T6, and may possibly be related to their content of hydroxymethylcytosine. If this substance, or the virus containing it, were to block, in the above scheme, either the production or the utilization of cytosine, a shunt of all nucleic acid synthesis into production of virus DNA, as is observed, would be the result. Just how such inhibition might be caused, however, we cannot at present say.

We have also noted (unpublished experiments) that the DNA of these viruses is more resistant to the action of deoxyribonuclease and phosphatase than is thymus DNA. This may possibly give the virus nucleic acid a selective advantage in the infected cell.

Finally, we may note that the presence of a different pyrimidine base provides a marker by which virus DNA may be distinguished from host DNA. This will be of use in determining, for example, whether virus DNA or its components are being synthesized during the first few minutes of infection of *Esch. coli* B by T2, when the net DNA content of the cell remains constant.

SUMMARY

1. The deoxyribonucleic acids of bacteriophages T2, T4 and T6 of *Esch. coli* contain no cytosine. Instead, they contain a hitherto unrecognized pyrimidine which has been isolated in crystalline form and found to be identical with synthetic 5-hydroxymethylcytosine.

2. The ultraviolet-absorption characteristics of 5-hydroxymethylcytosine are described, and those of its deamination product, 5-hydroxymethyluracil, partially described.

3. 5-Hydroxymethylcytosine is lost during hydrolysis of deoxyribonucleic acid with concentrated perchloric acid at 100°, but can be recovered almost quantitatively after hydrolysis with formic acid at 175°, provided that a sufficient volume is used. Yields of other bases, especially guanine, are also somewhat affected by the proportion of formic acid used.

4. The quantitative purine and pyrimidine com-

position of deoxyribonucleic acid from phages T2, T4 and T6, each in r and r^+ mutants, has been determined, and no differences could be detected among these viruses. The molar ratios adenine/thymine and guanine/5-hydroxymethylcytosine are close to unity and the ratio (adenine + thymine)/(guanine + 5-hydroxymethylcytosine) is 1·8.

5. No 5-hydroxymethylcytosine could be detected in any of the following materials: dried cells of *Esch. coli*, ox-spleen deoxyribonucleic acid, phages T5 and T7, an insect polyhedral virus, vaccinia virus and meningo-pneumonitis virus. Quantitative analyses of deoxyribonucleic acid bases from phage T5 and from vaccinia virus are reported.

6. The possible significance of 5-hydroxymethylcytosine, which may be a component peculiar to certain viruses, is discussed in relation to the metabolism of pyrimidine bases and of nucleic acids during virus infection.

We are indebted to a number of persons, as acknowledged in the text, for gifts of materials, and are especially grateful to Dr J. M. Sprague, Dr Bettylee Hampil and other members of the staff of Sharp & Dohme Inc., Research Division, for generous co-operation with the use of their facilities. The work of one of us (S. S. C.) was conducted under a grant from the Commonwealth Fund. This paper is Contribution no. 94, Division of Forest Biology, Science Service, Department of Agriculture, Ottawa, Canada.

REFERENCES

Bergold, G. (1947). *Z. Naturf.* **2**b, 122.
Bergold, G. H. (1953). *Advanc. Virus Res.* **1**, 91.
Bergold, G. & Pister, L. (1948). *Z. Naturf.* **3**b, 406.
Chargaff, E. (1951). *Fed. Proc.* **10**, 654.
Chargaff, E., Lipshitz, R., Green, C. & Hodes, M. E. (1951). *J. biol. Chem.* **192**, 223.
Cohen, S. S. (1947). *Cold Spr. Harb. Symp. quant. Biol.* **12**, 35.
Cohen, S. S. (1951). *Bact. Rev.* **15**, 131.
Cohen, S. S. & Arbogast, R. (1950*a*). *J. exp. Med.* **91**, 607.
Cohen, S. S. & Arbogast, R. (1950*b*). *J. exp. Med.* **91**, 619.
Davidson, D. & Baudisch, O. (1925). *J. biol. Chem.* **64**, 619.
Elwyn, D. & Sprinson, D. B. (1950). *J. Amer. chem. Soc.* **72**, 3317.
Goldthwait, D. A. & Bendich, A. (1952). *J. biol. Chem.* **196**, 841.
Herriott, R. M. & Barlow, J. L. (1952). *J. gen. Physiol.* **36**, 17.
Hershey, A. D. & Chase, M. (1952). *J. gen. Physiol.* **36**, 39.
Hitchings, G. H., Elion, G. B., Falco, E. A. & Russell, P. B. (1949). *J. biol. Chem.* **177**, 357.
Hoagland, C. L., Smadel, J. E. & Rivers, T. M. (1940). *J. exp. Med.* **71**, 737.
Laland, S. G., Overend, W. G. & Webb, M. (1952). *J. chem. Soc.* p. 3224.
Litzinger, A. & Johnson, T. B. (1936). *J. Amer. chem. Soc.* **58**, 1936.
Markham, R. (1953). *The Nature of Virus Multiplication*, ed. P. Fildes & W. E. van Heyningen, Cambridge University Press.
Marshak, A. (1951). *Proc. nat. Acad. Sci., Wash.*, **37**, 299.
Marshak, A. & Vogel, H. J. (1951). *J. biol. Chem.* **189**, 597.
Putnam, F. W. (1952). *Exp. Cell Res. Suppl.* **2**, 345.
Reichard, P. & Estborn, B. (1951). *J. biol. Chem.* **188**, 839.
Rutten, F. J., Winkler, K. C. & de Haan, P. G. (1950). *Brit. J. exp. Path.* **31**, 369.
Siminovitch, L. & Rapkine, S. (1952). *Biochim. biophys. Acta*, **9**. 478.
Smith, J. D. & Wyatt, G. R. (1951). *Biochem. J.* **49**, 144.
Tamm, C., Hodes, M. E. & Chargaff, E. (1952). *J. biol. Chem.* **195**, 49.
Watson, J. D. & Crick, F. H. C. (1953). *Nature, Lond.*, **171**, 737.
Weed, L. L. & Cohen, S. S. (1951). *J. biol. Chem.* **192**, 693.
Wyatt, G. R. (1951*a*). *Biochem. J.* **48**, 481.
Wyatt, G. R. (1951*b*). *Biochem. J.* **48**, 484.
Wyatt, G. R. (1952*a*). *Exp. Cell Res. Suppl.* **2**, 201.
Wyatt, G. R. (1952*b*). *J. gen. Physiol.* **36**, 201.
Wyatt, G. R. & Cohen, S. S. (1952). *Nature, Lond.*, **170**, 1072.
Wyatt, G. R. & Cohen, S. S. (1953). *Ann. Inst. Pasteur*, **84**, 143.

The Synthesis of Lactose from Glucose in the Mammary Gland

By O. K. REISS and J. M. BARRY*
Department of Biochemistry, University of Chicago

(*Received* 30 *May* 1953)

The mammary gland of goats and cows has been shown to absorb glucose from the blood stream during lactation (Folley, 1949), and lactose is formed when slices or suspensions of the mammary glands of guinea pigs and rats are incubated with glucose (Malpress & Morrison, 1950; Reithel, Horowitz, Davidson & Kittinger, 1952; Caputto & Trucco, 1952; Hills & Stadie, 1952). This work strongly suggested that lactose is synthesized from glucose absorbed from the blood stream by the mammary gland, and a recent experiment with [1-^{14}C]glucose did in fact prove that in the goat the glucose and galactose residues of lactose are derived, at least partly, from blood glucose (Barry, 1952*a*). This experiment, however, did not show quantitatively the significance of this synthesis of lactose from glucose; the possibility remained that the bulk of the lactose in milk had been synthesized from some unlabelled compound taken from the blood.

In the present experiments, uniformly labelled glucose was injected intravenously into a lactating goat, and blood and milk samples were taken at intervals up to 12 hr. after injection. The specific activities of blood glucose, of lactose, and of the glucose and galactose residues of lactose, were measured. The results show that blood glucose was the principal source of both the glucose and galactose residues of lactose.

EXPERIMENTAL

Radioactive glucose. Glucose labelled uniformly with ^{14}C was obtained from the U.S. Atomic Energy Commission. In order to test its purity, carrier glucose was added and two derivatives, phenyl-D-glucosazone and phenyl-D-glucosotriazole, were prepared and purified by recrystallization. The specific activities of these derivatives, after correcting for their content of unlabelled carbon, were within 10% of that of the original glucose, indicating that at least 90% of its radioactivity was due to glucose. Paper chromatography of the original material with aqueous phenol as a solvent (Partridge, 1948) gave only one radioactive spot, in the position of glucose.

In order to determine the distribution of activity in the molecule, the glucose was partially degraded by yeast fermentation: carbon atoms 3 and 4 were obtained as CO_2, while the ethanol from carbon atoms 1, 2, 5 and 6, was oxidized and isolated as silver acetate. The specific activities of these two groups of carbon atoms were identical with that of the original glucose, and with those of its derivatives after correcting for their content of unlabelled carbon.

Procedure in experiments on goat. The labelled glucose was dissolved in a few ml. of water and injected into the left jugular vein of a lactating goat which was giving about 1 l. of milk a day. The methods of obtaining blood plasma and milk samples were the same as described previously (Barry, 1952*b*).

Measurement of specific activities

Plasma glucose. Blood plasma glucose was fermented with baker's yeast and the CO_2 formed was carried in a stream of N_2 into NaOH solution; the procedure was adapted from methods of determining blood glucose by fermentation (Holden, 1937; Conway, 1950), and gives carbon atoms 3 and 4 of glucose as CO_2 (Koshland & Westheimer, 1950). The CO_2 was precipitated as $BaCO_3$ which was centrifuged on to the base of a standard aluminium cup to give a layer of uniform thickness. The sample was counted for sufficiently long to reduce the probable error to less than 3% and, after correction to infinite thickness, the specific activities were calculated using a factor previously determined with standard samples.

Lactose. A very convenient procedure was devised for isolating lactose from milk, based on the industrial method of Leviton (1949). The milk was centrifuged, the fat-free milk poured off from the cake of fat, and then freeze-dried. The resulting powder was added slowly with shaking to 85% (v/v) methanol at 10–15°, using 10–15 ml./g. of powder. The mixture was shaken for 5 min., centrifuged to remove protein, and the supernatant filtered through a sintered glass funnel (fine porosity), the filtration being completed within 15 min. of adding the powder to the methanol. The filtrate was acidified to pH 3–4 with 5N-HCl, left at room temperature with occasional shaking for 48 hr., and then left at 0° for a further 48 hr.; crystals began to appear within 5–10 hr. at room temperature. The crystals were filtered off, washed with ice-cold 70% (v/v) ethanol, and recrystallized by dissolving in hot water and adding ethanol to make 70% (v/v) ethanol; after 48 hr. at 0° the crystals were filtered off, washed and dried. This procedure yielded 60–70% of the lactose in milk as colourless crystals with a nitrogen content of less than 0·1%. Colorimetric assay with the anthrone reagent (Morris, 1948) indicated that the material was pure. The lactose was burnt in a stream of O_2 and the CO_2 converted into $BaCO_3$ and counted as before.

Glucose residue of lactose. Lactose was hydrolysed to glucose and galactose by heating with 0·01N-HCl in a sealed test tube in an autoclave at 15 lb./in.^{2} for 2 hr. (Ramsdell & Webb, 1945). The hydrolysate was neutralized and the specific activity of carbon atoms 3 and 4 of the glucose determined by fermentation with yeast as described above. Under the conditions used, the yeast did not ferment galactose.

* Present address: Department of Agriculture, University of Oxford.

Galactose residue of lactose. The galactose residue of lactose was converted into mucic acid by oxidation of the lactose with HNO_3. The product was reprecipitated twice from hot water and gave colourless crystals (m.p. 206°); the specific activity was determined after combustion to CO_2. From one of the mucic acid samples the di-*p*-bromophenacyl ester of mucic acid was prepared and purified by recrystallization; its specific activity, after correcting for its content of unlabelled carbon, was identical with that of the original mucic acid.

RESULTS

In Fig. 1 are shown the specific activities of lactose and of its glucose and galactose residues in five milk samples taken at intervals after the intravenous injection of uniformly labelled glucose into a lactating goat. The results of a second similar experiment are given in Table 1. It can be seen that, within the limits of experimental error, the specific activities of the glucose and galactose residue of each lactose sample were the same.

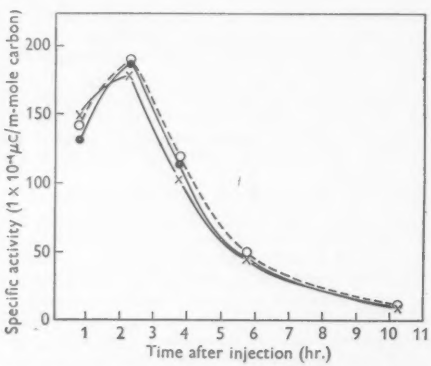


Fig. 1. Expt. 1. Specific activities of lactose and its glucose and galactose residues in milk samples taken at intervals after injecting 14 μc of uniformly labelled glucose intravenously into a lactating goat. The specific activities of each sample are plotted at the time half-way through the period in which the milk was secreted. O---O, lactose; ●—●, galactose; ×—×, glucose.

Blood plasma samples were also taken during these experiments, and the relation between the specific activities of blood glucose and milk lactose in Expt. 2 is shown in Fig. 2; the results of expt. 1 were similar. It has been assumed here that the blood glucose has remained uniformly labelled throughout the experiment, and that the specific activity of carbon atoms 3 and 4 which was actually measured was equal to the specific activity of the whole molecule; that this assumption is justified is suggested by the results in Table 1, which show that in the glucose residue of each lactose sample the specific activity of carbon atoms 3 and 4 was equal to the specific activity of all six carbon atoms. It can be seen that the specific activity of the lactose, after it had risen to a maximum at 1–2 hr. after the injection, was always equal to the specific activity of the blood glucose about 1–1·5 hr. earlier. During

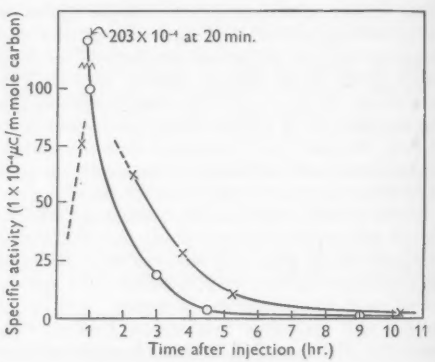


Fig. 2. Expt. 2. Uniformly labelled glucose (6 μc) injected intravenously into a lactating goat. The specific activity of each lactose sample is plotted at the time half-way through the period in which the milk was secreted; the curve drawn through these points gives approximately the specific activity, at any time, of lactose which has just reached a point in the mammary gland from which it can be drawn off by milking. O—O, blood glucose; ×—×, milk lactose.

Table 1. *Specific activities of lactose and its glucose and galactose residues after intravenously injecting 6 μc of uniformly labelled glucose into a lactating goat*

Experiment 2

Time of sampling (hr. after injection)	Specific activity (1×10^{-4} μc/m-mole carbon) Lactose	Galactose*	C-3 and C-4 of glucose†	Glucose‡
1·5	79·3	76·3	76·0	82·3
3	63·3	63·2	60·5	63·4
4·5	29·4	27·1	27·7	31·7
6	10·5	10·8	10·7	10·2
12	2·05	2·28	1·79	1·82

* Measured as mucic acid. † Obtained by fermentation. ‡ Calculated from lactose and galactose values.

the last few hours of the experiment the specific activity of the blood glucose remained almost constant, and was approximately equal to the specific activity of the lactose. The curves are similar to those found previously for the specific activities of lysine, tyrosine, and inorganic phosphorus in blood plasma, and the lysine, tyrosine and phosphorus of casein in milk (Barry, 1952*b*).

From the results in Fig. 2 it may be concluded that glucose was the principal compound taken from the blood stream by the mammary gland for the synthesis of lactose and that glucose carbon took, on the average, about 1–1·5 hr. to pass from the blood stream to a point in the mammary gland from which it could be drawn off in the lactose of milk. While glucose may have been the only compound used in the synthesis, the possibility cannot be excluded that a small proportion of the lactose came from unlabelled blood constituents. The possibility that some or all of the radioactivity in the lactose arose, not from glucose absorbed from the blood, but from some compound in the blood formed by the degradation of the injected glucose has been virtually excluded by a previous experiment performed under similar conditions (Barry, 1952*a*). It was then found that when [1-^{14}C]glucose was infused intravenously into the same goat and milk collected at 1·5 and 3·5 hr. after the end of the infusion, none of the radioactivity in the lactose was contained in carbon atoms 4, 5 and 6 of the glucose and galactose residues. Had the radioactive glucose been first degraded to C_3 or C_2 compounds and these then taken from the blood stream for the synthesis of lactose, it is highly improbable that this result would have been obtained.

The fact that the specific activities of the glucose and galactose residue of each lactose sample were identical shows that equal amounts of blood-glucose carbon were used by the mammary gland in the synthesis of both the glucose and galactose residue, and that there was no appreciable time lag between the appearance of blood-glucose carbon in the glucose and in the galactose residue. This agrees with the results of French, Popják & Malpress (1952), who also found equal specific activities in the glucose and galactose of lactose after feeding [^{14}C]starch by stomach tube to lactating rabbits. However, after infusing [1-^{14}C]glucose into a goat the specific activity of the galactose residue was about 30% lower than that of the glucose (Barry, 1952*a*); and recently Schambye, Wood & Popják (1953) have reported different distributions of activity in the glucose and galactose of lactose after administering various labelled compounds to lactating rabbits. Therefore, although our experiments show that blood glucose is the principal precursor of both the glucose and galactose residue of lactose in the goat, these results suggest that the synthesis of galactose has a more complex mechanism than a direct conversion of the glucose carbon chain into that of galactose.

SUMMARY

1. Glucose labelled uniformly with ^{14}C was injected intravenously into a lactating goat and the specific activities of blood glucose, of lactose, and of the glucose and galactose residues of lactose were measured in blood and milk samples taken up to 12 hr. after the injection.

2. The results show that blood glucose was the principal source of both the glucose and galactose residues of the milk lactose.

REFERENCES

Barry, J. M. (1952*a*). *Nature, Lond.*, **169**, 878.
Barry, J. M. (1952*b*). *J. biol. Chem.* **195**, 795.
Caputto, R. & Trucco, R. E. (1952). *Nature, Lond.*, **169**, 1061.
Conway, E. J. (1950). *Microdiffusion Analysis and Volumetric Error*, p. 220. 3rd ed. London: Crosby, Lockwood and Son.
Folley, S. J. (1949). *Biol. Rev.* **24**, 316.
French, T. H., Popják, G. & Malpress, F. H. (1952). *Nature, Lond.*, **169**, 71.
Hills, A. G. & Stadie, W. C. (1952). *J. biol. Chem.* **194**, 25.
Holden, R. F. (1937). *J. biol. Chem.* **119**, 347.
Koshland, D. E. & Westheimer, F. H. (1950). *J. Amer. chem. Soc.* **72**, 3383.
Leviton, A. (1949). *Industr. Engng Chem. (Anal.)* **41**, 1351.
Malpress, F. H. & Morrison, A. B. (1950). *Biochem. J.* **46**, 307.
Morris, D. L. (1948). *Science*, **107**, 254.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Ramsdell, G. A. & Webb, B. H. (1945). *J. Dairy Sci.* **28**, 677.
Reithel, F. J., Horowitz, M. G., Davidson, H. M. & Kittinger, G. W. (1952). *J. biol. Chem.* **194**, 839.
Schambye, P., Wood, H. G. & Popják, G. (1953). *Fed. Proc.* **12**, 264.

Alkaline Phosphatase of Milk

1. ASSOCIATION OF THE ENZYME WITH A PARTICULATE LIPOPROTEIN COMPLEX

By R. K. MORTON*
Department of Biochemistry, University of Cambridge

(*Received* 4 *June* 1953)

In order to obtain a highly purified alkaline phosphatase for detailed studies of this enzyme, especially in regard to possible transferase activity, milk was selected as a source of enzyme which might have some advantages over animal tissues. It is known that the enzyme of animal tissues is intimately associated with insoluble cellular particles (Kabat, 1941; Hers, Berthet, Berthet & de Duve, 1951). However, there was no evidence to suggest such an association of the enzyme with insoluble particles in milk. It therefore seemed possible that the autolytic (Albers & Albers, 1935) or tryptic (Schmidt & Thannhauser, 1943) procedures found necessary for release of the enzyme from animal tissues might be avoided by use of milk as the source of enzyme.

Although a phosphatase was recognized in milk by Demuth (1925), and later by Wilson & Hart (1932) and Graham & Kay (1933), the enzyme attracted little interest until Kay & Graham (1933) showed that the survival of this enzymic activity in pasteurized milk could be used as a sensitive method of detecting unsatisfactory heat treatment. Because of the importance of this 'phosphatase test' in the dairying industry, the relative heat-stability of the milk alkaline phosphatase has received considerable attention, as shown in reviews by Kay, Aschaffenburg & Mullen (1949), Sanders (1949) and Sjöström (1949). Comparatively few studies have been concerned with other properties of the enzyme.

Kay & Graham (1933) found that a portion of the phosphatase in milk was associated with the fat-globule membrane, from which it was released into the buttermilk by churning separated cream. A preparation of the fat-globule membrane from cream obtained by Hansson, Solberg & Sjöström (1946) was found to have considerable phosphatase activity. However, it seemed probable that much of the phosphatase activity remained in the separated milk after removal of the fat globules, since Zittle & Della Monica (1950) achieved some purification of the phosphatase using whey obtained from separated milk. These latter workers employed tryptic digestion in their purification procedures.

* Present address: Department of Biochemistry, University of Melbourne, Australia.

With such limited information available, it was clearly necessary to examine the relationship of the alkaline phosphatase to the other milk components. The distribution of the enzyme among the major milk fractions was therefore investigated. The results of these studies are reported in this paper.

It has been found that the milk alkaline phosphatase is firmly bound to insoluble particles of a lipoprotein nature. A method for almost quantitative separation of the enzyme from these particles, by the controlled use of *n*-butanol, has been developed. By this means, the milk phosphatase has been obtained in true solution and this has permitted purification as described in the following paper (Morton, 1953*a*). A brief account of the use of *n*-butanol for the purification of milk alkaline phosphatase and other enzymes has already been published (Morton, 1950).

MATERIALS AND METHODS

Enzyme unit. The unit of activity is defined as the amount of enzyme which liberates from sodium β-glycerophosphate, 1 μg. of inorganic phosphate P/min. at 38°. The specific activity is expressed either as units/mg. of protein N, as determined under optimal conditions, or, to facilitate comparison with published preparations, as Q_p values, i.e. the equivalent μl. of P liberated by 1 mg. of protein in 1 hr. at 38° (Engelhardt & Lyubimova, 1939). The factor 6·25 has been used throughout this work to convert protein N into equivalent dry weight.

Alkaline phosphatase activity. For the standard test, 1 ml. of the appropriately diluted enzyme preparation was added to 9 ml. of the buffered substrate at 38°, the final concentrations of the reactants being as follows: sodium β-glycerophosphate, 0·02 M; sodium veronal buffer, pH 9·8 at 38°, 0·04 M; $MgCl_2$, 0·01 or 0·001 M (see below). For estimation of activity of milk or milk products, or of crude enzyme preparations, the concentration of $MgCl_2$ was 0·001 M, higher concentrations being inhibitory. For all purified enzyme preparations, the concentration was 0·01 M.

The reaction was stopped after the appropriate interval by the addition of 5 ml. of 25% (w/v) trichloroacetic acid (TCA). Any precipitate was removed by filtration through no. 42 Whatman paper. Inorganic phosphate was then determined on a suitable sample of the filtrate and washings, and on a similar sample from a control tube stopped at zero time.

Because of the high phosphate blank, the amount of untreated milk, or of milk products, was adjusted to give approximately 10% hydrolysis of the substrate in 15 min. at 38°. For all more purified enzyme preparations, the amount of enzyme was adjusted to give hydrolysis of less than 5% of the substrate in 5 min. at 38°. The initial velocity thus determined was found to be proportional to the amount of enzyme.

Butter was tested by holding a weighed quantity at 38° until separation of the butterfat as an oil. The material was centrifuged, the cloudy serum removed with a syringe connected to a fine capillary, the fat washed with distilled water, and a sample from a known volume of mixed serum and washings used for determination of activity.

With the more highly purified enzyme preparations obtained in the later stages of the work, no protein precipitate was obtained with either TCA or with the molybdate-sulphuric acid reagent of Fiske & Subbarow (1925). Therefore, the volume of the final reactants was reduced to 5 ml. and the reaction stopped directly with the acid molybdate reagent, adjusted as to acid strength to allow for the alkali content of the buffer (see Delory & King, 1943). Inorganic phosphate was then estimated directly.

Inorganic orthophosphate. This was determined by the method of Fiske & Subbarow (1925).

Organic phosphate. This was determined by ashing a suitable small sample (about 0·2 ml.) with 0·5 ml. of 10N-H_2SO_4, and adding one drop of conc. HNO_3 to the charred material while hot, to complete oxidation. After being cooled and diluted with 2 ml. of distilled water, the tubes were heated on a boiling-water bath for 10 min. to hydrolyse any pyrophosphate. Then 1 ml. of 2·5% (w/v) ammonium molybdate was added, the contents were mixed and held for 5 min. and the colour was finally developed by addition of reducing reagent and adjustment to a final volume of 10 ml.

Protein nitrogen. The micro-Kjeldahl method was used. Dialysis in cellophan sacs against distilled water was used where necessary to remove non-protein N. The maximum absorption in the range 270–280 mμ. was also taken as a measure of protein concentration in later studies. The values were converted into protein nitrogen by frequent calibration with micro-Kjeldahl determinations.

Dry weight. The material was dried in a vacuum over conc. H_2SO_4 for 48 hr.

Lipid. This was initially estimated as the loss in dry weight following repeated extraction of the TCA precipitate, by centrifuging with 30% (v/v) ether in ethanol, at room temperature. Later, *n*-butanol was substituted for ether: ethanol, following observations to be described, and preliminary acid precipitation was omitted.

Butterfat of milk and dairy products was estimated essentially by the Gerber method (see Ling, 1945). Owing to lack of standardized equipment for this method the estimations were kindly carried out by the laboratory staff at the Milk Marketing Board Creamery, Norwich.

pH. All pH values were determined with the glass electrode, and are given for a temperature of 20°, unless otherwise indicated.

Solvents. Unless otherwise indicated, solvents used were of laboratory reagent grade.

RESULTS

The alkaline phosphatase activity of normal whole milk

There is a considerable variation in the activity of normal milk, as shown from the results in Table 1.

The mean value for two large bulks of milk show fair agreement with the mean of milk supplied from a small mixed herd, and may be considered as fairly representative. The increased activity with advanced lactation confirms the earlier findings of Folley & Kay (1936–7).

Distribution of activity among milk components

In order to establish the quantitative distribution of the phosphatase among the major components, milk was separated into several fractions as follows:

A sample (1 l.) of milk, obtained immediately after milking, was heated to 38° and centrifuged in 250 ml. cups for 45 min. at 2000 *g*. The cream, skim milk and sediment were carefully collected as different fractions by cooling the centrifuge cups and contents to 5° for 2 hr., and then mechanically removing the solidified cream layer. The separated milk was carefully collected by suction through a fine-glass tube and the sedimented material dispersed in

Table 1. *The alkaline phosphatase activity of normal whole milk*

(Sources of milk: (1) Pooled night and morning milk, supplied between September 1949 and April 1950 by a retail producer milking a mixed herd. (2) Milk from a small experimental herd. This was obtained through the courtesy of Prof. Beveridge and Dr W. McLymont, of the Department of Animal Pathology, University of Cambridge. (3) Milk from two groups of cows taken on the one day, kindly supplied by the retail producer. (4) Samples kindly supplied by the Milk Marketing Board Creamery, Norwich, each taken from 2000 gal. of pooled milk.)

Source	Mean* activity (units/ml.)	Mean* specific activity (Units/mg. N)	Q_p
(1) Commercial herd milk	11·9±0·5 (18)	2·5±0·18 (5)	17·3
(2) Experimental herd milk	28·3±4·3 (6)	3·9±0·13 (6)	26·6
(3) Commercial herd milk			
(*a*) cows in advanced lactation	14·0 (1)	.	.
(*b*) cows in early lactation	8·0 (1)	.	.
(4) Pooled milk	14·0 (2)	2·9 (2)	20·5

* Figures in brackets denote number of samples.

distilled water. Of the separated milk fraction, 300 ml. were then centrifuged in 50 ml. cups in a Servall-SS1 high-speed centrifuge at 14000 *g* for 30 min.

The small, surface-fat layer, the yellow translucent serum A, and the solid deposit of sedimented casein and other material (precipitate A) were again separately collected. The casein deposit was dispersed in distilled water, giving a stable white suspension. The serum A was re-centrifuged under the same conditions for 2·5 hr., and the precipitate (precipitate B) again separated from the yellow supernatant serum B and dispersed in distilled water.

The cream obtained from the first centrifuging was cooled and held at 5° for 14 hr. and then shaken in a glass jar on a reciprocating shaking machine until small butter granules appeared. The cloudy 'buttermilk' was carefully decanted, the butter granules pressed free of liquid with a spatula, the butter washed by 'working' it with distilled water, and the washings added to the buttermilk.

Phosphatase activities were determined at all stages. The results shown in Table 2 are calculated for an initial 1 l. of whole milk and are representative of those obtained with three different milk samples.

About 30 % of the alkaline phosphatase is removed with the fat globules in the cream fraction separated at low centrifugal force. Neither adjustment of the pH of the whole milk over the range 4·6–9·5 nor addition of a cationic detergent (cetyltetramethylammonium bromide, 0·02 % at pH 6·6) made any substantial difference to the distribution of the activity between the cream and separated-milk fractions. The activity of the initial whole milk was very much depressed following treatment at the lower pH values at which some precipitation of casein occurred. Repeated washing of the cream (at pH 6·8) by centrifuging (2000 *g*, 30 min.) with an equal volume of distilled water at 18° removed about 70 % of the activity in the cloudy aqueous layers. As shown in Table 2, about 65 % of the activity of the cream fraction is recovered in the buttermilk when the phase-inversion occurs on churning. There appears to be some destruction of enzyme during the churning process, presumably through surface denaturation.

Preliminary fractionation of separated milk

It is clear from Table 2 that over 50 % of the phosphatase activity of whole milk remains in the fat-free component, and hence it seemed desirable to examine possible methods of separating the enzyme from the major protein constituents, casein and β-lactoglobulin, as an essential preliminary to purification. As a general procedure for these experiments, whole milk was heated to 38°, centrifuged (1500 *g*, 45 min.), and the cream removed and churned. The buttermilk so obtained was added to the separated milk, and the mixed milk recentrifuged. The product was a fat-free milk of enhanced phosphatase activity, containing about 70 % of the original enzyme of the whole milk.

Sedimentation of casein. The results shown in Table 2 indicated that separation of the coarser casein particles (precipitate A), representing about 30 % of the casein of the milk, removed less than 6 % of the alkaline phosphatase.

In attempting to apply these findings on a larger scale, separated milk was centrifuged at 25° in two continuous-flow centrifuges, one having a multi-disk bowl, speed 6000 rev./min., the other a hollow bowl, speed 23000 rev./min. With each centrifuge a flow rate of approximately 200 ml./hr. gave a yellow opalescent serum, the combined sera having a specific activity of 2·5 units/mg. nitrogen. The sedimented casein was collected as a faintly opalescent, gelatin-like ribbon from the inside of the centrifuge bowls. It readily dispersed in distilled water to give a completely stable suspension.

Although some 10 l. of milk were fractionated by this method, effecting some purification of the enzyme, the method was slow and tedious, and

Table 2. *Distribution of alkaline phosphatase activity among milk components*

Treatment	Fractions obtained	Activity of fractions: Vol. (ml.)	Units/ml.	Total units	Protein N (mg./ml.)	Specific activity (units/mg. N)	Recovery (% whole milk)	Purification
Whole milk, 2000 *g*, 45 min.	Whole milk	1000	13·4	13 400	4·9	2·7	100	1
	Separated milk	935	8·1	7 574	4·8	1·7	56·5	0·6
	Cream	68	60·0	4 080	2·1	28·6	30·4	10·6
	Sediment	8	6·7	54	.	.	0·4	.
Separated milk, 14 000 *g*, 30 min.	Serum A	922	7·3	6 731	2·9	2·2	50·2	0·8
	Ppt. A	160	4·6	736	13·6	0·3	5·5	0·1
	Fat	10	23·0	230	.	.	1·7	.
Serum A, 14 000 *g*, 2 hr. 30 min.	Serum B	870	4·6	4 002	2·7	1·7	29·9	0·6
	Ppt. B	30	83·0	2 490	11·6	7·2	18·6	2·7
Cream, shaken	Buttermilk	28	91·0	2 548	4·4	20·7	19·0	7·7
	Butter*	15	27·4	411	.	.	3·1	.

* Figures expressed in terms of emulsion obtained at 38°.

ultimately considered impracticable for large-scale purification work.

Effect of pH. The pH was adjusted from the normal (pH 6·7) with 0·2 N sodium hydroxide up to pH 10·9, and with 0·2 N acetic, lactic and hydrochloric acids to pH 4·4, at two temperatures (0 and 18°). The results were essentially the same with the three acids and at both temperatures. After centrifuging (approx. 5000 *g*, 15 min. using the high-speed attachment, International no. 1 centrifuge), the supernatant was adjusted to pH 7·5, and any precipitate was rubbed into distilled water and adjusted to pH 8·0 to favour dispersion of the casein particles. There was little change in activity over the pH range 5·5–9·5, but rapid loss of activity below pH 5·0 and above pH 10·0 was observed. At pH above 9·0, the milk became progressively more viscous and yellow owing to swelling and dissolving of casein particles. With the decline in pH from 5·5 to 4·6, there was increasing precipitation of casein (until complete at the isoelectric point, pH 4·6), and progressive loss of enzyme activity. No purification was obtained by casein precipitation in this way.

Fractionation with salts. In general, sufficient solid was added to separated milk, maintained at pH 6·5–7·0 by appropriate additions of N sodium hydroxide or N hydrochloric acid, until visible precipitation of the casein complex occurred. After standing for 30 min. to 1 hr., at controlled temperature, the milk was either filtered through fluted paper (Whatman no. 1) or centrifuged (1500 *g*, 30 min.). The precipitates were dispersed in distilled water and dialysed free of salt before testing. The filtrates were treated similarly. Slow filtration through the precipitated protein gave clear filtrates, which were invariably devoid of activity, although slight activity was sometimes found in the opalescent supernatants obtained by centrifuging. Most of the enzyme, however, was associated with the casein precipitate. In no case was there any purification of the enzyme.

Precipitation of casein with the following salts used at neutral pH and at the percentage saturation indicated (in brackets) gave less than 10 % total recovery of the enzyme: NaCl (satd.); $(NH_4)_2SO_4$ (60 %); $MgCl_2$, $12H_2O$ (satd.); $CaCl_2$, $6H_2O$ (satd.); anhyd. $MgSO_4$ (satd.) and anhyd. Na_2SO_4 (satd.). However, when the casein was precipitated by saturation with K_2HPO_4 and NaH_2PO_4, added in the proportions required to keep the pH at 6·6, about 40 % of the total activity was recovered. The casein obtained by this procedure was much more flocculent and more readily dispersed in water than that obtained with any other salt. It was also observed that the addition of 5 g. Na_2HPO_4/100 ml. milk, prior to precipitation of the casein with NaCl or $CaCl_2$, $6H_2O$, considerably improved the recovery of enzyme but sodium citrate was not similarly effective. The addition of phosphate did not alter the low recovery of enzyme obtained when ammonium sulphate was used to precipitate the casein.

Fractionation using rennin and pepsin. Separated milk at 37° was adjusted to pH 6·0 with N hydrochloric acid after addition of 2 ml. of M calcium chloride per 100 ml. milk. Sufficient rennin or pepsin was added to cause a firm clot in 10 min. The coagulum was cut with a sharp knife into small cubes, the yellow, slightly turbid whey decanted from the curd at regular intervals over 1 hr., and then centrifuged. The whey was adjusted to pH 7·5, and the paracasein precipitate or 'curd' was dispersed by grinding in a mortar with distilled water and adjusting to pH 8·0, before testing. The results are shown in Table 3.

Addition of 10 % (v/v) calcium phosphate gel (32 mg. dry weight/ml.) to rennin whey (pH 5·8), previously dialysed against running tap water (4 hr.) and distilled water (4 hr.), caused adsorption of most of the enzyme. However, while repeated elution of the precipitate with 10 % ammonium sulphate solution at pH 8·0 recovered about 20 % of the activity, the extract was opalescent and there was no purification. Centrifuging the dialysed whey (at pH 5·5) at 14000 *g* for 1 hr. gave a small upper layer of fat and clear supernatant of low activity, while the precipitate contained most of the enzyme.

Following digestion at pH 8·8, by addition of 0·01 % crude trypsin and incubation for 18 hr. at 25°, a sample of the same whey was adjusted again

Table 3. *Alkaline phosphatase activity of fractions obtained with rennin and pepsin*

Treatment conditions					
pH	Precipitation with	Fraction	Specific activity (units/mg. N)	Recovery* (% whole milk)	Purification
6·5	Rennin	Milk	1·32	100	1
		Curd	0·3	15	0·3
		Whey	2·6	41	2·0
6·0	Pepsin	Milk	3·90	100	1
		Curd	0·2	8	0·1
		Whey	7·8	62	2·0

* Low total recoveries are due to occlusion of alkaline phosphatase by the paracasein precipitate (see text).

to pH 5·8 and centrifuged as before. Approximately 65 % of the total activity now remained in the supernatant, indicating considerable modification of the enzyme association by this treatment. This result suggested a similarity in behaviour between the alkaline phosphatase in milk, and that reported for the enzyme in animal tissues (Kabat, 1941).

Fractionation with acetone. The methods and the apparatus described by Askonas (1951) for fractionation of soluble enzymes of muscle were used. It was found that the optimum pH for fractionation of dialysed separated milk was in the range pH 6·3–6·6. Magnesium acetate (final concentration 0·015 M) was used to adjust the ionic strength and gave better results than did sodium acetate. In the absence of sufficient magnesium (or calcium) ions, difficulty was experienced in centrifuging down the casein precipitate.

The form of the enzyme recovery curve obtained with acetone fractionation of separated milk and based on a number of separate experiments is shown in Fig. 1. Small changes in the conditions of treatment did not markedly affect the form of the curve (curve 1), but the use of sodium phosphate (0·008 M), at pH 6·6, to adjust the ionic strength markedly delayed precipitation of the enzyme. This is indicated by the distinctly different form of the enzyme recovery curve (curve 2) as shown in Fig. 1. The effect is similar to that reported by Askonas (1951) for muscle proteins, although more marked in this case. This may be partly due to the interaction of inorganic phosphate with the casein complex.

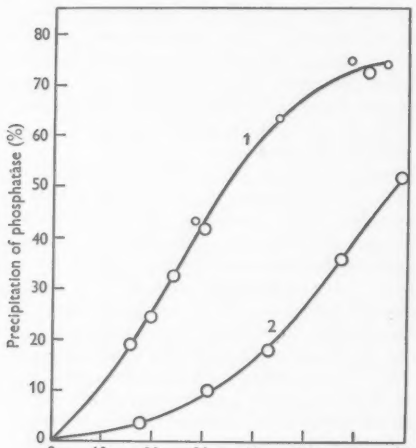


Fig. 1. Effect of acetone concentration on recovery of alkaline phosphatase during fractionation of dialysed separated milk. Curve 1, initial concentration of magnesium acetate, 0·015 M; pH 6·4. Curve 2, initial concentration of sodium phosphate, 0·008 M; pH 6·6.

The casein fraction of the milk proteins was precipitated at acetone concentrations between 35 and 50 % (v/v), and the greater part of the enzyme accompanied this fraction. As shown in Table 4, however, there was some small purification of the enzyme in the higher acetone fraction, but the yield was low. All fractions with enzymic activity were either distinctly cloudy, or opalescent, as had been found in fractionation studies using salts.

The high enzyme recoveries obtained during acetone fractionation of separated milk contrast markedly with those obtained using either acids or salts. The acetone-precipitated casein readily dispersed in distilled water, and gave completely stable suspensions, resembling closely the behaviour of native, undenatured casein obtained by sedimentation. This was in marked contrast to the products obtained with acid and salts, excepting with sodium phosphate.

In a single experiment, separated milk was fractionated with acetone at room temperature (20°). There was very low recovery of the phosphatase (total recovery about 20 %), and the casein was resistant to dispersion.

These observations have indicated a close relationship between the extent of denaturation of casein and the phosphatase activity of the casein fraction. The findings suggest that casein prepared by acid precipitation is already largely denatured, and hence the product so extensively studied by protein chemists may be considerably different from the native protein.

It was found that ethyl ether (A.R. grade), previously cooled to − 15° and added to the dialysed milk at 0° to give a final concentration of 9·5 % (v/v) prior to acetone fractionation, considerably improved the total recovery of the enzyme since much less acetone was required for precipitation. The use of ether, added before addition of acetone, has

Table 4. *Alkaline phosphatase activity of fractions obtained with acetone*

Treatment conditions					
pH	Precipitation with acetone	Fraction	Specific activity (units/mg. N)	Recovery (% whole milk)	Purification
6·5		Milk	2·02	100	1
	0–46 %, v/v	Ppt.	1·15	49	0·6
	46–65 %, v/v	Ppt.	5·64	27	2·8

been found to improve the fractionation of the enzyme during later purification stages and has been adopted as a useful modification of the acetone-fractionation procedure for general use.

However, the addition of ether, up to 20 % (v/v), together with acetone, did not alter the evident association of the phosphatase with particulate components of the milk.

Preparation of an enzymically active lipoprotein complex

On the basis of the results obtained during the above preliminary investigations, and combining several procedures as shown in Table 5, a 43-fold purification of the phosphatase from 10 l. of separated milk was obtained. The dialysed product of stage 6 (termed preparation A) was an almost colourless, faintly opalescent solution. When frozen and dried in vacuum over sulphuric acid, it gave a white powder, which was readily taken up in dilute buffer (0·02 M veronal-hydrochloric acid), pH 7·5, giving an apparent solution.

When adjusted to pH 4·6 only faint turbidity appeared, and preparation A was therefore considered essentially free of casein. However, when dialysed against distilled water (12 hr.) and centrifuged at 14000 *g* for 45 min., 82 % of the activity was found in the white precipitate. While considerably purified, the enzyme was not in true solution.

Analysis indicated that preparation A contained about 10 % lipid, which appeared to be largely phospholipid (lipid P, 3·2 %). Trypsin digestion of rennin whey had indicated that some enzyme remained in true solution following this treatment. These two observations suggested that phosphatase in milk was associated with a lipoprotein complex, which had been considerably purified as preparation A. A number of lipid solvents were therefore examined as means of disrupting this complex.

Removal of lipid with butanol. Samples (5 ml.) of the opalescent dispersion of preparation A, containing 1·5 mg. protein nitrogen/ml., and at pH 7·5 in veronal-hydrochloric acid buffer (0·01 M), were treated by stirring and gently shaking (30 sec.) with excess of organic solvent or bile salt at room temperature (18°). After centrifuging (approx. 5000 *g*, 30 min.) and carefully removing the aqueous layer with a fine capillary attached to an all-glass syringe, the aqueous layer was suitably diluted and tested for phosphatase activity immediately. Table 6 records the activity and the qualitative appearance of the aqueous material after removal of excess of solvent and dilution with an equal volume of distilled water. In contrast to the opalescent or turbid extracts obtained with all other organic solvents or bile salts, both *n*- and *iso*butanol yielded water-clear, colourless aqueous extracts with good recovery of the initial activity. The remainder of preparation A was therefore treated similarly with *n*-butanol. The clear aqueous layer was removed, filtered through a thin layer of Hyflo on a Büchner funnel, dialysed against distilled water until free from butanol (18 hr.), and dried over sulphuric acid in a vacuum. The resultant material (termed preparation B) was completely soluble in distilled water, and no precipitate appeared after centrifuging for 2 hr. at 14000 *g*. No further lipid could be extracted from the trichloroacetic acid precipitate with 30 % (v/v) ether in ethanol, indicating that the single extraction with *n*-butanol removed virtually all lipid material.

Examination of whole milk and products, prepared as previously described, showed that in all cases *n*-butanol disrupted the lipoprotein complex as effectively as in the case of preparation A, releasing the bound alkaline phosphatase. In order to precipitate the casein complex in the same treatment, the following procedure was developed. The material (at pH 7·5) was treated with excess of (20 %, v/v) *n*-butanol and stirred vigorously or shaken (30 sec.). After heating to 38°, the pH was adjusted to 5·2 with N acetic acid, and the emulsion held for a further 5 min. at 38° before centrifuging. The water-clear aqueous layer was separated and

Table 5. *Partial purification of phosphatase from separated milk*

(Stages of preparation: (1) Initial whole milk, pH 6·7. (2) Milk separated, dialysed, and adjusted pH 6·3. (3) Precipitate obtained at 0–46 % (v/v) acetone at −2°. (4) Precipitate dialysed, adjusted to 0·01 M calcium acetate and to pH 5·7. Heated, 40°, 15 min., in litre batches. (5) Precipitate with ether (8 %, v/v) and further acetone (0–18 %, v/v). (6) Dialysed and repeated stage 4. Centrifuged 3500 *g*, 30 min., pH 7·2. Dried in vacuum. 'Preparation A'. (7) Preparation A in 20 ml. water, at pH 7·5, shaken (20 secs.) with 10 ml. *n*-butanol and held 38°, 5 min. at pH 5·2. Aqueous layer dialysed and dried in a vacuum. 'Preparation B'.)

Stage of preparation	Vol. (ml.)	Activity (units/ml.)	Total (units × 10^3)	Protein N (mg./ml.)	Specific activity (units/mg. N)	Yield (%)	Purification
(1)	10 000	20·1	201·0	5·11	3·9	100	1·0
(2)	8 900	12·1	108·0	3·52	3·4	54	0·9
(3)	3 940	13·2	52·0	6·20	2·1	26	0·5
(4)	4 190	9·9	41·5	0·81	12·3	21	3·2
(5)	400	41·3	16·5	0·83	49·6	8	12·7
(6)	390	29·6	11·6	0·22	134·5	6	34·5
(7)	15	446·0	6·7	0·48	930·0	3	238·0

Table 6. *Effect of lipid solvents on alkaline phosphatase activity*

	Material treated*			
	Preparation A		Cream	
Solvent or bile salt	Enzyme recovery (%)	Relative† turbidity	Enzyme recovery (%)	Relative† turbidity
(Untreated)	100	+ + +	100	+ + + +
Ether:ethanol (1:3)	45	+ +	N.D.	N.D.
n-Butanol	82	–	76	–
*iso*Butanol	68	–	66	–
n-Amyl alcohol	N.D.	N.D.	75	+ +
Ethyl acetate	69	+ +	N.D.	N.D.
Carbon tetrachloride	82	+ + +	69	+ + + +
Chloroform	68	+ + +	53	+ + +
Toluene	88	+ +	72	+ + +
Deoxycholate (1%)	72	+ +	N.D.	N.D.
Taurocholate (1%)	94	+ +	N.D.	N.D.
Petroleum ether	N.D.	N.D.	85	+ + +

* Preparation A treated at pH 6·8, cream at pH 5·5.

† Scale of relative turbidities of aqueous phase after treatment and dilution with equal volume distilled water is as follows: opaque, + + + +; opalescent, + +; clear, –; not determined, N.D.

brought to pH 7·5. In all cases it contained between 50 and 70% of the initial phosphatase in true solution. The results obtained with organic solvents in comparative trials with cream at pH 5·5 are shown in Table 6. Similar results were obtained with both milk and buttermilk. Improved yields obtained with dialysed milk indicated that low ionic strength was desirable during treatment of enzymes with *n*-butanol.

The combined procedures giving preparation B, and summarized in Table 5, yielded a very active enzyme, completely water-soluble and purified 238 times when compared with the enzyme in normal milk. However, such is the activity of milk phosphatase that preparation B must be considered less than 5% pure. These preliminary experiments, however, provided the basis of the method for obtaining the highly purified enzyme.

DISCUSSION

The low specific activity of normal cow's milk (2·5 units/mg. N) indicates that it compares very unfavourably as a source of alkaline phosphatase with animal tissues. Untreated dispersions of bovine kidney and intestinal mucosa, for example, have specific activities of the order of 20–25 and 70–100 units/mg. nitrogen respectively (Morton, 1953*b*) while Abul-Fadl, King, Roche & Thoai (1949) have reported values of 100–135 and 650–800 units/mg. nitrogen respectively for autolysates of these tissues.

In contrast to most animal tissues, however, the major proteins of milk have been fairly well defined, and the enzymes present seem limited in number (see review by McMeekin & Polis, 1949). Hence it appeared quite probable that alkaline phosphatase would be readily separable from other constituents by application of standard techniques. The failure of such methods can undoubtedly be attributed to the binding of milk phosphatase in a particulate complex.

Hitherto, milk has been considered to contain fine particles belonging to three categories, namely: (1) coarse, readily sedimentable foreign matter, such as udder cells, and leucocytes; (2) fat globules surrounded by a protein membrane; (3) colloidal casein particles (the casein-calcium phosphate complex). The fractionation of milk by differential centrifuging (Table 2) clearly showed that little or no alkaline phosphatase was associated with the coarse material of category 1. However, up to 30% of the enzyme was associated with the fat globules, most of which could be removed by centrifuging and collection of the cream layer.

Kay & Graham (1933) first observed the higher phosphatase activity of cream compared with milk, and more recently Kay *et al.* (1949) have directed attention to it in relation to tests for adequate pasteurization. However, there has been no previous quantitative estimate of the amount of activity associated with the milk fat. Following careful removal of the cream, the separated milk is found to contain the remaining 70% of the phosphatase activity, but it is probable that the smaller fat globules in this phase are more efficient in adsorbing enzyme than are the larger globules obtained in the cream layer. However, between 50 and 60% of the total phosphatase activity of whole milk is undoubtedly associated with the fat-free phase, although, as later work has shown, the enzyme is not present in true solution.

The results confirm that the enzyme associated with the fat phase is attached to the fat-globule membrane (Kay & Graham, 1933; Sjöström, 1940), probably by an adsorption mechanism, since repeated washing of the fat elutes the enzyme into the turbid aqueous extract. This evidence is now supported by electron micrograph studies (Morton, 1953*c*). It is possible that all the phosphatase enters the milk attached to the fat globules and largely dissociates from the membrane in the milk cistern and during subsequent handling, especially during centrifuging. There has been no investigation to decide between this and the alternative hypothesis that some of the particulate enzyme is adsorbed after secretion of the fat globule.

The enzyme is most conveniently recovered from the fat phase by churning to butter, release of the enzyme accompanying the phase inversion. In this process some of the smaller fat globules do not coalesce, but escape into the buttermilk. Between 10 and 15 % of the activity of the buttermilk is associated with these small globules. It has been confirmed that the washed butterfat produced by churning has no activity (Kay & Graham, 1933).

Because of the increased phosphatase activity and lower protein content, cream (and its aqueous washings) and buttermilk all have higher specific activity than milk (Table 2). However, fractionation studies have indicated that buttermilk behaves very much the same as separated milk, so that the essential association of the enzyme with particulate matter, as discussed below, is not altered by adsorption on the fat globules in milk.

The predominant particles of the separated milk are those of category 3 above, namely colloidal particles of the casein-calcium phosphate complex. Centrifuging (14 000 *g*, 30 min.) deposits the coarser particles from separated milk as a translucent, faintly blue fraction (Table 2, precipitate A), without significant removal of the phosphatase. The small precipitate (Table 2, precipitate B), obtained by longer centrifuging (14 000 *g*, 2 hr. 30 min.) is similar in appearance but contains some phosphatase activity, so that the specific activity of this fraction is 2·7 times that of the original milk. It is clear, therefore, that in normal milk the enzyme is attached to particles capable of sedimentation with a definite fraction of the casein particles.

The results obtained with chemical fractionation studies are consistent with the attachment of the phosphatase to a distinct particulate component and indicate further that the complex is not dissociated by normal fractionation procedures.

As would be expected for a particulate protein fraction, precipitation of the enzyme occurs in the pH range 4·6–5·2, and at comparatively low salt and low acetone concentrations. In all such fractionation studies, the water-clear supernatants were uniformly devoid of activity.

The marked loss in activity at pH 4·6, the isoelectric point of casein, and in all cases with salt fractionation, warrants further consideration. As shown in the following paper (Morton, 1953*a*), the partially purified phosphatase in true solution shows only small loss of activity over the range pH 4·6–7·5, although marked irreversible denaturation occurs close to pH 4·4. Furthermore, the enzyme in true solution was frequently fractionated with ammonium sulphate with quite normal recovery (70–80 %). Thus there occurs the very unusual phenomenon of an enzyme having apparently greater stability when highly purified than when examined in the crude source material.

The explanation appears to be connected with the particulate nature of the enzyme in milk. Differential centrifuging (Table 2) indicates that particles carrying phosphatase activity must be of about the same size and in intimate association with the exceedingly more numerous casein particles. This has now been confirmed by studies with the electron microscope (Morton, 1953*c*). Under conditions favouring co-precipitation and change in the casein complex, there is some occlusion of the particulate enzyme so that it is no longer accessible to the substrate during the activity test. Hence treatments which change the physical state of the precipitated casein alter the relative accessibility of enzyme and substrate and so change the estimate of activity. Thus the readily dispersable casein precipitate obtained with mixed phosphates gave higher enzyme recovery than the markedly granular precipitate obtained with ammonium sulphate. The relative rate of acidification has also been found to alter the physical state of casein precipitated near the isoelectric point and similarly also the phosphatase activity. This is no doubt the explanation of the variable effect of acidification reported by Sjöström (1949) but ascribed by him as due to other causes. Sjöström (1949) considered his earlier evidence to support the reversible dissociation of phosphatase into co-enzyme and apo-enzyme, but found difficulty in explaining results of later studies.

While occlusion of the particulate phosphatase is the major factor, there is some evidence that the particles carrying the enzyme are themselves modified to some extent by low pH and by salts, and that this may affect their apparent enzymic activity. While the enzymically active particles are largely co-precipitated with casein during acetone fractionation, the casein remains undenatured and readily dispersable to form stable suspensions in distilled water. Such dispersions show their full enzymic potential in the activity test and, when treated with butanol, the bound enzyme is released into true solution. However, butanol treatment of

acid- or salt-precipitated casein is not so effective in releasing the bound enzyme. This further suggests that the casein thus precipitated may have undergone partial denaturation.

The specific precipitation of casein as paracasein (Table 3) effects some purification, leaving much of the particulate-bound enzyme largely in the whey fraction. This procedure was not adopted because of the desire to avoid proteolytic enzymes of any type. It has been used as a basic procedure for milk phosphatase purification by Zittle & Della Monica (1950), who obtained a 60-fold purification of the enzyme as compared with the activity in separated milk. These workers appreciated that the enzyme was associated with lipid material, which remained attached to the enzyme, even following vigorous treatment with chloroform. Zittle & Della Monica (1951) reported difficulties in elution of milk phosphatase adsorbed on 'Celite' from an opalescent preparation obtained following tryptic digestion of whey, and compared their observations with those reported for certain particle-bound enzymes and certain virus preparations. These workers have applied the butanol method (Morton, 1950) for the purification of milk phosphatase from separated milk and have confirmed the unique action of this solvent (Zittle & Della Monica, 1952).

Although Kekwick, Mackay & Record (1946) found ether valuable for fractionation of human blood plasma and preparation of fibrinogen, ether alone did not precipitate any protein from milk under the experimental conditions employed. However, the addition of ether (10 %, v/v) prior to fractionation of milk with acetone has been found to give both higher recoveries of enzyme and some improvement in fractionation. It is noteworthy that Martland & Robison (1929) employed a mixture of ether and ethanol at room temperature for precipitation and fractionation of bone phosphatase, but the present investigation appears to be the first study of the use of ether and acetone together for low-temperature fractionation of enzymes.

The enzymically active material isolated as preparation A was shown to be a lipoprotein complex. Treatment of the aqueous opalescent suspension of preparation A with *n*- or *iso*butanol was found to extract the lipid material (mostly phospholipid) and to release simultaneously the alkaline phosphatase into true solution (Table 6). Butanol was similarly effective in releasing the enzyme into true solution directly from milk, cream and other milk products (Table 6).

The immediate change in the properties of the alkaline phosphatase following butanol treatment confirms the hypothesis that the phospholipid is responsible for the binding of the enzyme in the particulate complex. This contention also gains support from the finding by Sjöström (1940) of a positive correlation between the phospholipid contents and alkaline phosphatase activities of various milk and milk fractions. More recently it has been verified by the isolation and examination of the enzymically active lipoprotein particles of cow's milk (Morton, 1953*c*).

Although the enzymic activity was found to be fairly stable to various organic solvents (Table 6) usually considered to be excellent lipid solvents, with the exception of butanol these were quite ineffective in disrupting the lipoprotein complex of preparation A sufficiently to release the bound enzyme into true solution. It is notewothy also that treatment of preparation A with ether at $-50°$, a procedure found to disrupt the lipoproteins of blood plasma (McFarlane, 1942), did not release the alkaline phosphatase into solution. Moreover, the enzyme was not obtained in solution when an acetone powder of preparation A was extracted with 0·85 % sodium chloride. These findings indicate a unique action of butanol which has been fully confirmed by later experience (Morton, 1950; Zittle & Della Monica, 1952).

SUMMARY

1. The alkaline phosphatase activity of normal cow's milk is very low as compared with organs such as kidney and intestine. The Q_p value is about 20.

2. About 30–40 % of the enzyme is adsorbed to the butterfat globules and may be concentrated by separation of cream. The enzyme is released into the aqueous phase (buttermilk) on churning cream to butter, or by elution with distilled water. The butterfat itself is devoid of activity.

3. The phosphatase in fat-free separated milk and buttermilk is not in true solution in the serum, but is associated with an insoluble particulate lipoprotein complex. This particulate material may be sedimented together with a portion of the casein particles by high-speed centrifuging.

4. Precipitation of casein from separated milk with acids, salts or organic solvents results in coprecipitation of the lipoprotein particles together with the associated phosphatase. The more specific precipitation of casein with rennin (or pepsin) permits of some separation of the casein and lipoprotein particles.

5. Precipitation of casein with acid and certain salts produces partial denaturation, resulting in occlusion of the phosphatase in the protein precipitate and apparent loss of enzymic activity. Inorganic phosphate partially protects casein from denaturation during salt precipitation.

6. The lipoprotein complex which binds milk alkaline phosphatase may be disrupted by treatment of the aqueous material (milk or its products) with

n- or *iso*butanol. Removal of the lipid is accompanied by simultaneous release of the enzyme into true solution. No other organic solvent tested was effective in releasing the bound phosphatase, indicating a unique action of butanol.

I wish to thank Dr M. Dixon, F.R.S., for his encouraging interest and advice during this work. This work was carried out while holding a Travelling Scholarship awarded by the Gowrie Scholarship Trust of Australia, whose assistance is gratefully acknowledged.

REFERENCES

Abul-Fadl, M. A. M., King, E. J., Roche, J. & Thoai, N-v. (1949). *Biochem. J.* **44**, 428.
Albers, H. & Albers, E. (1935). *Hoppe-Seyl. Z.* **232**, 165.
Askonas, B. A. (1951). *Biochem. J.* **48**, 42.
Delory, G. E. & King, E. J. (1943). *Biochem. J.* **37**, 547.
Demuth, F. (1925). *Biochem. Z.* **159**, 415.
Engelhardt, W. A. & Lyubimova, M. N. (1939). *Nature, Lond.*, **144**, 668.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Folley, S. J. & Kay, H. D. (1936–7). *Enzymologia*, **1**, 48.
Graham, W. R. & Kay, H. D. (1933). *J. Dairy Res.* **5**, 54.
Hansson, E., Solberg, P. & Sjöström, G. (1946). *Svenska Mejeritidn.* **38**, 191.
Hers, H. G., Berthet, J., Berthet, L. & Duve, C. de (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 21.
Kabat, E. A. (1941). *Science*, **93**, 43.
Kay, H. D., Aschaffenburg, R. & Mullen, J. E. C. (1949). *12th Int. Dairy Congr. Papers and Communications*, **2**, 743.
Kay, H. D. & Graham, W. H. (1933). *J. Dairy Res.* **5**, 63.
Kekwick, R. A., Mackay, M. E. & Record, B. A. (1946). *Nature, Lond.*, **157**, 629.
Ling, E. R. (1945). *A Textbook of Dairy Chemistry*, vol. 2, 2nd ed. London: Chapman and Hall.
McFarlane, A. S. (1942). *Nature, Lond.*, **149**, 439.
McMeekin, T. L. & Polis, B. D. (1949). *Advanc. Protein Chem* **5**, 202.
Martland, M. & Robison, R. (1929). *Biochem. J.* **23**, 237.
Morton, R. K. (1950). *Nature, Lond.*, **166**, 1092.
Morton, R. K. (1953*a*). *Biochem. J.* **55**, 795.
Morton, R. K. (1953*b*). *Biochem. J.* (submitted for publication).
Morton, R. K. (1953*c*). *Biochem. J.* (submitted for publication).
Sanders, G. P. (1949). *12th Int. Dairy Congr. Papers and Communications*, **2**, 757.
Schmidt, G. & Thannhauser, S. J. (1943). *J. biol. Chem.* **149**, 369.
Sjöström, G. (1940). *Svenska Mejeritidn.* **32**, 139.
Sjöström, G. (1949). *12th Int. Dairy Congr. Papers and Communications*, **2**, 750.
Wilson, L. T. & Hart, E. B. (1932). *J. Dairy Sci.* **15**, 1.
Zittle, C. A. & Della Monica, E. S. (1950). *Arch. Biochem.* **26**, 112.
Zittle, C. A. & Della Monica, E. S. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 193.
Zittle, C. A. & Della Monica, E. S. (1952). *Arch. Biochem.* **35**, 321.

Alkaline Phosphatase of Milk

2. PURIFICATION OF THE ENZYME

By R. K. MORTON*
Department of Biochemistry, University of Cambridge

(*Received* 4 *June* 1953)

It has been shown in the previous paper (Morton, 1953*a*) that alkaline phosphatase in milk is intimately associated with insoluble lipoprotein particles. In this respect the enzyme resembles the alkaline phosphatases of animal tissues (Kabat, 1941; Hers, Berthet, Berthet & Duve, 1951), but unlike the tissue enzymes (see Roche & Thoai, 1950) milk phosphatase is only partly released from the particulate material when this is treated with trypsin or lipase (Zittle & Della Monica, 1952).

A far more effective method of obtaining the phosphatase in true solution, and one which avoids undesirable exposure of the enzyme to proteolysis, is the treatment of the whole milk or milk product with butanol (Morton, 1950, 1953*a*). Using this procedure to obtain the enzyme in true solution, and by further treatments, the alkaline phosphatase has been highly purified. The final product is considered to be essentially homogeneous protein. The details of the method are described in this paper.

* Present address: Department of Biochemistry, University of Melbourne, Australia.

METHODS

The analytical methods have been described in the previous paper (Morton, 1953*a*).

Preparation of activated charcoal. Animal charcoal (British Drug Houses) (180 g.), was treated successively with 5 l. of 0·1 N-HCl, 2 l. 5% KCl, 200 ml. 0·1 M-$Ba(OH)_2$, and finally with 2 l. of 5% KCl. After each treatment the charcoal was sucked dry over no. 42 Whatman paper on a large Büchner funnel. After the last treatment it was dried at 100° in an oven, and stored in an air-tight jar. I am indebted to Dr P. D. Mitchell for suggesting this procedure for obtaining an active charcoal, free of inorganic phosphate.

EXPERIMENTAL AND RESULTS

Preliminary studies

Because of the very low specific activity of whole milk (Morton, 1953*a*) it was clear that very large bulks of material would have to be treated in order to obtain sufficient of the enzyme to enable effective purification.

Preliminary experience in the treatment of a large bulk was obtained using 33 l. of cream (71% butterfat) obtained by commercial separation of 500 l. of whole milk. After dilution with water to 43 l., it was adjusted to pH 5·5 and treated with *n*-butanol (3·5 l./6 l. cream) at 38° for 5 min. The emulsion was centrifuged (1800 *g*, 30 min.) in 1 l. glass cups in an International Serum Centrifuge, and the clear, greenish, butanol-saturated aqueous layer (which contained the phosphatase in true solution) was collected by suction. The enzyme was precipitated with acetone, adsorbed from the dialysed solution on to calcium phosphate gel (prepared essentially as described by Keilin & Hartree, 1951), and eluted with 0·1 M-$NaHCO_3$. This gave a brown solution from which the enzyme was obtained by addition of solid $(NH_4)_2SO_4$, taking the fraction between 65 and 85% saturation (at pH 7·0). The clear colourless solution so obtained had a specific activity of 3050 units/ml., representing a purification of 985 times as compared with the initial whole milk, and an overall yield of 2%. Efforts to obtain further purification by a wide variety of procedures were unrewarding. However, electrophoretic examination in 0·033 M phosphate buffer, pH 8·0 showed at least three proteins to be present.

A large number of small-scale trials, using a variety of procedures, were carried out with the partially purified enzyme (specific activity 3050 units/ml.) obtained from cream. The results of these studies provided the basis for the method of purification of the enzyme described in the following section. They also serve to indicate some of the properties of the milk phosphatase, and therefore are briefly summarized here.

Effect of pH. The pH of a sample of the enzyme solution of specific activity 3050 units/ml. was carefully and progressively lowered at room temperature, by slow addition of 0·05 N acetic acid. There was no visible precipitation of any protein, although a very faint opalescence appeared at about pH 4·5, and no purification was gained by this procedure. There was no change in appearance by adjustment to alkaline pH values. The results shown in Table 1 indicate the difference in the recovery of the enzyme when in true solution and when bound by particles.

Effect of heating. Heating a sample of the same enzyme solution gave the results shown in Table 2.

Adsorption. A large number of adsorbents were examined, including calcium phosphate gel, magnesium phosphate gel, alumina gel (Cγ), animal charcoal, glass dust, Hyflo Supercel (Johns-Manville and Co.), kieselguhr, kaolin of various grades, magnesium and calcium carbonates, diphenylcarbinol (Neumann, 1949) and salicylic acid. Particularly at pH values about 5·0–5·5, the enzyme can be adsorbed by addition of sufficient of most of these materials. Only calcium phosphate gel, alumina gel, and specially prepared charcoal deserved further examination. Over the pH range 5·0–8·5, the enzyme can be adsorbed and then readily eluted from calcium phosphate and alumina gel, although more difficulty is experienced in eluting the enzyme from charcoal. $NaHCO_3$ (0·1 N), or 5% $(NH_4)_2SO_4$ or Na_2SO_4 at pH 8·0 are all equally effective for elution of the enzyme. However, adsorption of inert protein, rather than of enzyme, generally gave more satisfactory results.

Table 1. *Effect of* pH *on milk alkaline phosphatase*

(Each preparation treated for 15 min. at 20° at pH indicated and then readjusted to pH 7·5 prior to testing at pH 9·8 (37°) using sodium β-glycerophosphate as substrate. Enzyme preparations: preparation 1 was dialysed, phosphatase-enriched, fat-free separated milk (Morton, 1953*a*). Preparation 2 was a partially purified enzyme preparation of specific activity 3050 units/mg. N, obtained in true solution by treatment of cream with butanol.)

	Relative activity	
pH (20°)	Preparation 1 (particle-bound enzyme)	Preparation 2 (enzyme in true solution)
9·5	96	22
9·0	96	67
7·5	100	100
5·0	60	96
4·7	38	80
4·6	27	68
4·4	5	15
4·3	5	5

Table 2. *Heat stability of milk alkaline phosphatase*

(Enzyme preparation: the partially purified enzyme, of specific activity 3050 units/ml., was used. The preparation, at pH 7·8, was heated for 2 min. at temperature indicated, and then rapidly cooled to 20°.)

Temp. (°)	Relative activity
45	102
50	91
55	76
60	46

Final purification method

Stage 1. Whole milk was commercially separated at 35° to give about 50 l. of cream of 69% butterfat.

Stage 2. After cooling to 10° and dilution with water to give 50% butterfat, the cream was churned in two wooden hand-churns, each of 5 gal. capacity. The buttermilk and two wash waters were combined and cooled to about 10°. Further treatment was carried out after transporting to the laboratory.

Stage 3. The buttermilk, at pH 6·6, was treated in batches, adding 2·4 l. (29%, v/v) of *n*-butanol to 6 l. of buttermilk, previously heated to 32°. The material was vigorously stirred and heated to 35° for approximately 5 min., and then adjusted to pH 4·95 by addition of 750 ml. of 0·25 N acetic acid. The casein was thus precipitated, and on centrifuging, the material separated into distinct layers comprising a light white precipitate, a clear yellow

aqueous zone overlaid by light particles, and a brown surface layer of excess of *n*-butanol, containing dissolved material.

Stage 4. After removing the excess of butanol, the serum was removed by suction through a narrow glass tube and filtered through a 1 cm. layer of Hyflo Super-cel over no. 1 Whatman paper on a large Büchner funnel. It was then adjusted to pH 8·5 with 0·2N sodium hydroxide and held overnight at 5°, when a bulky white precipitate settled. The supernatant was largely decanted and the remainder centrifuged to remove the precipitate.

Stage 5. The clear yellow-green serum, adjusted to pH 6·3, was treated in batches by adding 10% (v/v) ether to the material at 0°, and precipitating the protein by the addition of a further 45% (v/v) of acetone, at −3 to −5°. The batches were held overnight at −7°, the supernatant carefully decanted, and the pink precipitate collected by centrifuging in a refrigerated centrifuge at −5°.

Stage 6. The precipitate, dissolved in a minimum of distilled water and filtered overnight at 0° through Whatman no. 542 filter paper, gave a cherry-red solution, completely water-clear. After dialysis to remove organic solvents, it was fractionated with ammonium sulphate at pH 7·1. The lower ammonium sulphate fraction (0–63% saturation) containing the red protein had little phosphatase activity and was discarded. The fraction which precipitated between 63 and 85% saturation of ammonium sulphate was pale straw-yellow in colour. After dialysis for 3 days against frequent changes of distilled water at 5°, it gave 127 ml. of protein solution of specific activity 4102 units/mg. nitrogen, a purification of 1520 times compared with the initial whole milk, with a yield of 6·5%.

Stage 7. The dialysed enzyme was adjusted to pH 8·6 with 0·1N sodium bicarbonate, diluted with water to 180 ml., and then treated by slow addition of 9 g. of treated animal charcoal, added as a slurry in distilled water. After thorough stirring for 30 min., the charcoal was removed by centrifuging and discarded. To the supernatant 12 ml. of alumina gel (C_γ, 22 mg. dry weight/ml.) was added, mixed, and the precipitate discarded.

Stage 8. The resultant supernatant of specific activity 7261 units/mg. nitrogen (purification factor, 2690 times) was dialysed for 2 hr. against distilled water at pH 7·5, adjusted to pH 6·4, and fractionated by addition of ether (10%, v/v) followed by acetone (40–49%, v/v), at −3°. The precipitate was dissolved and dialysed against distilled water to remove organic solvents, and centrifuged at 14000 *g* for 20 min.

Stage 9. The supernatant was readjusted to pH 8·6, 5 g. of treated charcoal was added, and centrifuged at 14000 *g* for 30 min.

Stage 10. The supernatant from centrifuging was immediately fractionated with ammonium sulphate at pH 7·2, largely to eliminate traces of charcoal apparently in stable colloidal suspension. The active fraction between 69 and 82% saturation of ammonium sulphate was dialysed at pH 7·5 to low ionic strength.

Stage 11. After adjusting to pH 6·5 and 0·015M magnesium acetate, and following addition of ether to 10% (v/v) at 0°, the dialysed solution was fractionated with acetone between 42 and 48% (v/v). After dialysis for 4 hr. against 5 l. of distilled water (adjusted to pH 7·5) at 0°, the product was dried in vacuum over sulphuric acid. The fine white powder so obtained had a specific activity of 15314 units/mg. nitrogen (Q_p, i.e. equivalent μl. P liberated by 1 mg. protein in 1 hr. at 38°, 106000), representing a purification of 5660 times compared with the initial whole milk on a protein nitrogen

Table 3. *Purification of milk alkaline phosphatase*

(Summary of stages: (1) Whole milk. (2) Buttermilk and washings from cream (69% butterfat). (3) Filtered aqueous layer following butanol treatment. (4) Filtrate obtained after treatment at pH 8·5, 12 hr., at 5°. (5) Red solution of precipitate obtained with ether (10%, v/v) and acetone (0·45%, v/v). (6) Yellow solution of precipitate obtained with $(NH_4)_2SO_4$, 63–85% saturation. (7) Supernatant from charcoal adsorption at pH 8·6. (8) Colourless solution of precipitate obtained with ether (10%, v/v) and acetone (40–49%, v/v). (9) Supernatant from second charcoal adsorption. (10) Fraction obtained with $(NH_4)_2SO_4$, pH 7·2, 69–82% saturation. (11) Final solution obtained with ether (10%, v/v) and acetone (42–48%, v/v).)

Stage	Vol. (ml.)	Activity (units/ml.)	Total (units × 10^3)	Protein N (mg./ml.)	Specific activity (units/mg. N)	Yield (%)	Purification
(1)	$1·3 \times 10^6$	13·9	18 070	5·10	2·7	100·0	1
(2)	32 500	80	2 600	2·80	28·6	14·4	11
(3)	27 120	66	1 790	0·21	314·3	9·9	116
(4)	27 000	65	1 755	0·20	325·0	9·7	121
(5)	1 480	1 092	1 616	1·20	910·0	8·9	337
(6)	127	9 230	1 172	2·25	4 102	6·5	1 520
(7)	280	3 340	935	0·46	7 261	5·2	2 690
(8)	75	9 800	735	1·04	9 423	4·1	3 490
(9)	89	5 730	510	0·49	11 690	2·8	4 330
(10)	12	29 000	348	2·04	14 200	1·9	5 270
(11)	10	26 800	268	1·75	15 314	1·5	5 660

basis. This corresponds to a purification of about 11 000 times on a non-dialysable dry-weight basis, or about 24 000 times on a total dry-weight basis. The final yield of purified enzyme was 1·5 %. All attempts at further purification beyond this stage caused a decline in the specific activity of the preparation, which was considered to be essentially pure milk phosphatase.

A summary of the purification is given in Table 3.

Electrophoretic studies

The preparations obtained during purification of the enzyme were examined at several stages, using a Tiselius electrophoresis apparatus. The material of specific activity 4102 units/mg. nitrogen (Table 3, stage 6) showed the presence of three protein components which separated in 0·033 M phosphate buffer, pH 8·0 (Fig. 1, upper photograph). The leading peak separated sufficiently after 1 hr. to enable removal of a sample without contamination with either of the other two proteins. The specific activity of this fast-moving peak was 192 units/mg. nitrogen, while that of the sample, which contained some of each of the other two remaining proteins, was 9600 units/mg. nitrogen.

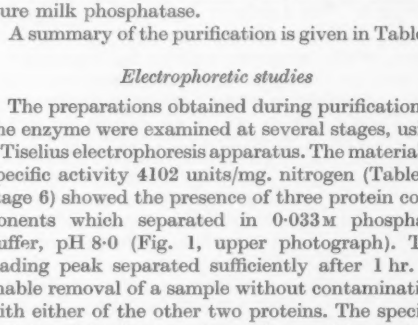


Fig. 1. Electrophoretic analyses during purification of milk phosphatase. The upper photograph shows material of specific activity 4102 units/mg. N (Table 3, stage 6) after 1 hr.; the lower photograph, material of specific activity 9423 units/mg. N (Table 3, stage 8) after 1 hr. 45 min. On the left are the ascending (anodic), and on the right the descending (cathodic) boundaries obtained with phosphate buffer (pH 8·0, 0·033 M) at 5·0 v/cm.

The material of specific activity 9423 units/mg. nitrogen (Table 3, stage 8) was examined under the same conditions. Only two proteins separated (Fig. 1, lower photograph). A sample of the leading protein gave a specific activity of 100 units/mg. nitrogen, while that of the slower-moving protein gave 12 000 units/mg. nitrogen. Since this protein showed no tendency to separate further, the activity of the sample should approximate to that of the pure enzyme.

The material of specific activity 9423 units/mg. was also examined in 0·125 M acetate buffer, pH 5·5. However, there was very little separation of the proteins, even after 2 hr.

Insufficient material remained after final purification (Table 3, stage 11) to permit of electrophoretic examination since the apparatus available required about 12 ml. of a 1 % protein solution.

DISCUSSION

When the alkaline phosphatase of milk has been released into true solution by disruption of the lipoprotein particles which bind the enzyme, it is capable of considerable purification by procedures of a more or less standard type. The essential step in the purification of this enzyme is the complete removal of the phospholipid fraction with which the enzyme is so intimately associated. This can be achieved only by treatment of the milk, or milk product, with *n*- or *iso*butanol, the butanol being unique in this reaction (Morton, 1950; Zittle & Della Monica, 1952).

While some disruption of the particulate component can be achieved by prolonged treatment of rennin-whey with trypsin, difficulties are experienced in later stages of purification. One of the most pronounced changes of milk phosphatase following butanol treatment is in the behaviour on adsorption and elution. Before butanol treatment, the enzyme is fairly readily adsorbed, but a small portion only may be regained by elution. After removal of the lipoprotein complex, the enzyme can readily be quantitatively recovered by proper elution. Zittle & Della Monica (1951, 1952) have also drawn attention to this change in behaviour following butanol treatment. In using the valuable adsorption method for enzyme purification, it is essential that the enzyme to be purified is in true solution and free of any associated lipid. Where difficulty is experienced in using the adsorption method, the presence of contaminant phospholipid should be suspected.

Although animal charcoal has been widely used for adsorption of non-protein materials, it has not commonly been employed in enzyme purification. Dixon & Kodama (1926) used charcoal for purification of xanthine oxidase, while Abul-Fadl & King (1949) used it for decolorization of a preparation of alkaline phosphatase from dog faeces. In the purification of milk phosphatase animal charcoal, treated as described to remove inorganic phosphate, has been found to be superior to other adsorbents for selective removal of inert protein. It also removed traces of compounds absorbing at 260 mμ., presumably nucleic acids or derivatives of them. It

may be found useful in the purification of enzymes other than alkaline phosphatase.

At stage 6 of the purification procedure (p. 797), the lower ammonium sulphate fraction contained a red protein, the identity of which has not been established. The colour was completely reduced on addition of sodium dithionite. The fraction showed some xanthine dehydrogenase activity, and the absorption spectrum was like that of xanthine dehydrogenase (see Morell, 1952). However, it is considered that the very strong red colour of this fraction could not arise from xanthine dehydrogenase alone, and it is probable that another red protein was present. It is noteworthy that Sørensen & Sørensen (1938–41) and Theorell & Åkeson (1943) have both described red proteins found in separated milk.

Although insufficient protein was recovered to enable electrophoretic examination of the final product, it had a specific activity (15314 units/mg. nitrogen) greater than that of the most active material separated by electrophoresis (specific activity, 12000 units/mg. nitrogen). Moreover, the final product was found to be a specific phosphomonoesterase, free of any diesterase or pyrophosphatase activities (see Morton, 1953*b*). Hence it is considered that the purified enzyme is essentially pure.

Since this work was carried out and briefly reported (Morton, 1950), Zittle & Della Monica (1952) have obtained a 1000-fold purification of milk phosphatase by using butanol treatment of rennin-whey from separated milk, followed by careful acetone fractionation and adsorption of non-active protein on 'Filter-cel'. This relative purification is based on a comparison with separated milk, which has a specific activity about 0·6 times that of whole milk (Morton, 1953*a*). On this basis, the purification factor for the product obtained in this study would be about 9500 times. It is not surprising, therefore, that Zittle & Della Monica (1952) found their final product showed several components when examined electrophoretically, and that the preparation had diesterase activity.

Caputto & Marsal (1941) obtained a partial purification of the alkaline phosphatase of cow mammary gland using prolonged autolysis followed by ethanol fractionation, a procedure introduced by Albers & Albers (1935) for purification of kidney phosphatase. The final product obtained by Caputto & Marsal (1941) had a specific activity equivalent to 3850 units/mg. N (Q_p 26800). Their preparation may be directly compared with the milk phosphatase of specific activity 15300 units/mg. nitrogen (Q_p 106000) obtained in the present study, since the milk phosphatase is derived from the mammary gland (Folley & Kay, 1936–7; Folley & White, 1936). Moreover, the milk and mammary gland alkaline phosphatases are identical (Morton, 1953*b*).

While milk itself has a very low specific activity (Morton, 1953*a*), it should be noted that buttermilk from high-fat cream is quite as active as some animal tissues (e.g. kidney and liver). Where available as an industrial by-product, raw-cream buttermilk therefore represents an inexpensive and convenient source from which purified alkaline phosphatase may be obtained with a yield of about 10% (see Table 3). From whole milk, of course, the yield is very much lower (1·5%, as in Table 3).

Although the preparation of milk alkaline phosphatase as described in this paper has not been repeated, there should be no great difficulty in reproducing these results. It is suggested that a useful modification would be to sediment the enzymically active lipoprotein particles from buttermilk by centrifuging at high speed (50000 *g* or greater), wash the particles free of soluble proteins as in the preparation of intestinal phosphatase (see Morton, 1950) and then to apply the procedures herein described. Such a modification is possible since equipment for high-speed centrifuging of relatively large quantities is now available, whereas it was still lacking in the period 1949–50 when the work described above was completed.

SUMMARY

1. The alkaline phosphatase of milk was purified 5600 times compared to the initial whole milk on a protein nitrogen basis. The yield was 1·5%. The details of the method are described.

2. An essential step in the purification procedure depends on the unique ability of butanol to disrupt the lipoprotein complex with which the phosphatase is normally associated.

3. The final product had a specific activity of 15300 units/mg. nitrogen (Q_p 106000), estimated from hydrolysis of sodium β-glycerophosphate at 37°. Comparison of the activity with a protein fraction separated electrophoretically indicated that the purified enzyme was probably homogeneous. Homogeneity was also indicated by the lack of diesterase or pyrophosphatase activity in the preparation.

4. A red protein fraction was separated from the buttermilk during the purification. The identity of this protein was not established.

I wish to thank Dr M. Dixon, F.R.S., for his encouraging interest and advice during this work, and particularly for carrying out the electrophoretic examinations of the milk proteins. I am grateful to the Manager of the Milk Marketing Board Creamery, Norwich, for his co-operation in supplying raw materials for this work. I also wish to record my thanks to Mr R. Brown for some technical assistance. This work was carried out while holding a Travelling Scholarship awarded by the Gowrie Scholarship Trust of Australia, whose assistance is gratefully acknowledged.

REFERENCES

Abul-Fadl, M. A. M. & King, E. J. (1949). *Biochem. J.* **44**, 434.
Albers, H. & Albers, E. (1935). *Hoppe-Seyl. Z.* **232**, 165.
Caputto, R. & Marsal, A. (1941). *Rev. Soc. argent. Biol.* **17**, 139.
Dixon, M. & Kodama, K. (1926). *Biochem. J.* **20**, 1104.
Folley, S. J. & Kay, H. D. (1936–7). *Enzymologia*, **1**, 48.
Folley, S. J. & White, P. (1936). *Proc. Roy. Soc.* B, **120**, 346.
Hers, H. G., Berthet, J., Berthet, L. & Duve, C. de (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 21.
Kabat, E. A. (1941). *Science*, **93**, 43.
Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
Morell, D. B. (1952). *Biochem. J.* **51**, 657.
Morton, R. K. (1950). *Nature, Lond.*, **166**, 4073.
Morton, R. K. (1953*a*). *Biochem. J.* **55**, 786.
Morton, R. K. (1953*b*). *Biochem. J.* (submitted for publication).
Neumann, H. (1949). *Biochim. biophys. Acta*, **3**, 117.
Roche, J. & Thoai, N-v. (1950). *Advanc. Enzymol.* **10**, 83.
Sørensen, M. & Sørensen, S. P. L. (1938–41). *C.R. Lab. Carlsberg*, **23**, 55.
Theorell, H. & Åkeson, A. (1943). *Ark. Kemi Min. Geol.* **17**B, 1.
Zittle, C. A. & Della Monica, E. S. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 193.
Zittle, C. A. & Della Monica, E. S. (1952). *Arch. Biochem. Biophys.* **35**, 321.

Oxidative Pathways in a Fluorescent *Pseudomonas*

By MARGOT KOGUT and E. P. PODOSKI

Agricultural Research Council Unit for Microbiology, Department of Microbiology, University of Sheffield

(*Received* 25 *April* 1953)

During an investigation of the oxidative metabolism of a strain of a fluorescent *Pseudomonas* (KB 1) it was noted that when succinate was oxidized by washed suspensions there was a sharp break in the oxygen-uptake curve corresponding to approximately 1·5 moles oxygen/mole succinate added. Examination of the suspending fluid for possible end products showed the presence of keto acids which were tentatively identified as pyruvate and α-ketoglutarate by partition chromatography. The production of α-ketoglutarate by micro-organisms has been reported by Lockwood & Stodola (1946) using a strain of *Ps. fluorescens*; by Dagley, Fewster & Happold (1952) using *Vibrio*, strain 01; by Hockenhull, Wilkin & Winder (1951) using *Penicillium chrysogenum*; by Walker, Hapton & Hall (1951) using *Aspergillus niger*; and by Hunter (1952) using *Mycobacterium butyricum*. Lockwood & Stodola (1946) showed that when their strain of *Ps. fluorescens* was grown upon a medium containing glucose and with a high carbon/nitrogen ratio, with carefully controlled aeration, up to 18 moles α-ketoglutarate/100 moles glucose consumed accumulated in the growth medium. Dagley *et al.* found that this keto acid was produced when *Vibrio* 01 oxidized either benzoate, phenylacetate, citrate, acetate or succinate, though the amounts formed were not determined.

The formation of α-ketoglutarate from succinate by *Ps. fluorescens* suggested that some, at least, of the reactions of the tricarboxylic acid cycle played a part in the oxidative metabolism of this organism. A detailed study was therefore made of the oxidation of succinate and other tricarboxylic-acid-cycle intermediates by this organism using whole cells and cell-free extracts. Since the oxidation of some of the substrates appeared to be adaptive, it was also possible to examine the application of the simultaneous adaptation technique (Stanier, 1947) to problems of this type. A preliminary account of this work has already been given (Kogut, 1952*a*, *b*).

MATERIALS AND METHODS

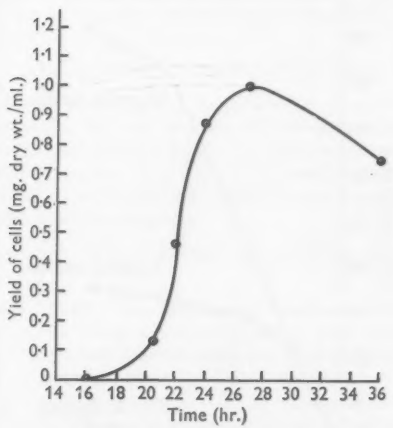
Organism. The organism was isolated from soil and had the following properties: Gram-negative motile rods 1·0–2·0 μ. × 0·4–1·0 μ.; with a single polar flagellum; a green, fluorescent pigment was produced which diffused into the growth medium; opt. temp. 30°, growth occurred at 37°; gelatin not liquefied; nitrites not produced from nitrates; catalase positive; indole not produced; litmus milk, no change. These properties are compatible with either *Ps. convexa* or *Ps. ovalis*. These two species are differentiated from one another by Bergey (1948) according to their temperature range. Thus *Ps. ovalis* has an optimum temperature of 25° with poor or no growth at 37°, whereas *Ps. convexa* is said to grow readily at 37° and to have an optimum temperature of 30°. For convenience we propose to use the term *Ps. fluorescens* species group.

Cultures were maintained in the dry state (Stamp, 1947), and when in use, were subcultured monthly on 10% (v/v) yeast autolysate (Barker & Beck, 1942) agar slopes. Unless otherwise stated, the organisms were grown on the following medium: K_2HPO_4, 0·1%; NH_4Cl, 0·1%; Mg_4SO_4, $7H_2O$, 0·05%; all (w/v), in distilled water and supplemented with the following trace elements: 100 μg./l. Fe^{2+} as $FeSO_4$.$(NH_4)_2SO_4$, $6H_2O$; 200 μg./l. B as H_3BO_3; 100 μg./l. Co^{2+} as

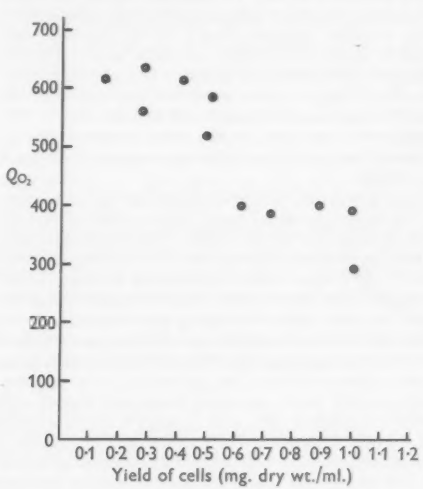
$CoSO_4$, $7H_2O$; 10 μg./l. Cu^{2+} as $CuSO_4$, $5H_2O$; 10 μg./l. Mn^{2+} as $MnSO_4$, $4H_2O$; 100 μg./l. Mo as $(NH_4)_6Mo_7O_{24}$, $4H_2O$; 200 μg./l. Zn^{2+} as $ZnSO_4$, $7H_2O$. Sodium succinate, sodium citrate or other single carbon source, as mentioned in the text, in concentration of 0·4% (w/v). The sterile medium was dispensed in 300 ml. portions in penicillin flasks, and after inoculation was incubated at room temperature (18–25°) with shaking. The inocula were prepared by suspending a loopful of stock culture in 5 ml. of the same medium as that to be used for large-scale culture and incubating at 30° for 5–6 hr. Preliminary investigations showed that the oxidative activity of washed suspensions varied with the growth stage of the organism at the time of harvesting (cf. Wooldridge, Knox & Glass, 1936; Gale & Stephenson, 1938, 1939). The most active suspensions, Q_{O_2} 500–600 (Q_{O_2} = μl. O_2/mg. dry wt./hr.), were obtained when cells were harvested before they had reached half the maximum yield for the medium in question (Fig. 1*a*, *b*). Inocula were therefore so adjusted that the activity after overnight growth (14–18 hr.) was in this range. Cells were harvested by centrifugation, washed twice with and suspended in distilled water to give a concentration of 3 mg. dry wt./ml. Dry weight was determined by measuring the optical density of a suitably diluted sample in the Hilger Spekker absorptiometer with a neutral grey filter Ilford no. H508, and referring to a standard graph relating optical density to dry weight.

Reagents. Reagents obtained from supply houses were A.R. grade. Acids were neutralized with NaOH before use. α-Ketoglutaric acid was prepared by the modified method of Clutterbuck (1927), as given by Umbreit, Burris & Stauffer (1949). Great difficulty was experienced in freeing this compound from small traces of succinate, using the method recommended by these authors. Pure samples in very low yield were finally obtained by repeated recrystallization from acetone and benzene. *cis*-Aconitic acid was prepared by the method of Malachowski & Maslowski (1928). DL-*iso*Citric acid was prepared by hydrolysis of a sample of trichloromethylparaconic acid, and assayed for the natural dextrorotatory *iso*citrate by the method of Ochoa (1948). All quantities of *iso*citrate given in this paper refer to the natural dextrorotatory form of the acid as above. A preparation containing approximately 15% triphosphopyridine nucleotide (TPN) and 30% diphosphopyridine nucleotide (DPN) was made from horse liver by the method of Lepage & Mueller (1949) and assayed for TPN by the method of Ochoa (1948) and for DPN by the method of Racker (1950). Adenosine triphosphate as the barium salt was prepared from rabbit muscle by the method of Lohmann (1931) as modified by Needham (1942). Coenzyme A was a preparation obtained from Armour laboratories and contained 10 Lipmann units/mg. Solutions of oxaloacetate were prepared from oxaloacetic acid immediately before use. 2:4-Dinitrophenylhydrazine obtained commercially was recrystallized from *n*-butanol.

Analytical methods. Manometric measurements were carried out in the conventional Warburg apparatus at 30°; the reactions were stopped by tipping 0·3 ml. 5N-H_2SO_4 from the second side arm. Succinic acid was estimated by the method of Krebs (1937). α-Ketoglutaric acid was



(*a*)



(*b*)

Fig. 1. (*a*) Variation of Q_{O_2} succinate (μl. O_2/mg. dry wt./hr.) with growth expressed as mg. dry wt./ml. of cells in the growth medium. Q_{O_2} calculated from the oxygen uptake during the period 5–35 min. after tipping. Manometer cups contained: in the side arm 0·5 ml. of bacterial suspension (1·5 mg. dry wt. of cells), in the main cup 0·5 ml. of 0·066M phosphate buffer pH 7·4, 0·6 ml. of 0·2M sodium succinate, distilled water to 2·3 ml., in the centre well 0·2 ml. of 20% (w/v) KOH. Temp. 30°. (*b*) Growth curve of cells grown on a mineral medium with 0·4% (w/v) sodium succinate at 25° with shaking. Yield of cells (mg. dry wt./ml.) obtained by measuring the optical density of a suitably diluted sample in the Spekker absorptiometer and referring to a standard graph relating dry weight to optical density.

estimated by the transaminase method of Krebs (1950); the spectrophotometric method of Goodwin & Williams (1952) was used for the estimation of α-ketoglutaric acid and of pyruvic acid.

When the transaminase method was used the acidified reaction mixture was transferred quantitatively to a graduated cylinder and heated in a boiling-water bath for 10 min. to decompose any oxaloacetate, since it reacts in this method; pyruvate does not interfere in this system. After adjusting the pH of the reaction mixture to 5·0, α-ketoglutarate was estimated in a sample. For the method of Goodwin & Williams, 0·5 ml. of the acidified reaction mixture was pipetted into a graduated cylinder, diluted to 5·0 ml. and any oxaloacetate present decomposed as described above. (Recoveries of α-ketoglutaric acid tended to be 5–10% low by this method and those of pyruvic acid correspondingly high.)

Keto acids were identified by paper-partition chromatography of the 2:4-dinitrophenylhydrazones after the method of Cavallini, Frontali & Toschi (1949). The developing solvent was *n*-butanol saturated with water and the papers (Whatman no. 1) were developed by the descending method.

Acidified manometer-cup contents were transferred to centrifuge tubes and the cells centrifuged off. To each of the supernatants were added 2 ml. of 0·1% (w/v) 2:4-dinitrophenylhydrazine in 2N-HCl and the mixture was kept at room temperature for 30 min. The solution was extracted three times with 2 ml. of ether and the pooled ethereal extracts were shaken up with 2 ml. of 10% (w/v) Na_2CO_3. The aqueous layer was removed, acidified and again extracted with ether (2 ml.). The ethereal extract was taken to dryness on the water bath and the residue dissolved in 0·5 ml. ethanol. Samples (50 μl.) of this solution were applied to sheets of Whatman no. 1 filter paper. The 2:4-dinitrophenylhydrazones of pyruvate and α-ketoglutarate were put through the same extraction procedure and the ethanolic solutions so obtained used as controls.

Respiratory quotients (R.Q.) were measured by the method of Dickens & Šimer (1930) as modified by Elsden & Lewis (1953).

Preparation of cell-free extracts. These were prepared either with the Hughes bacterial press (Hughes, 1951) or with the Mickle disintegrator (Mickle, 1948). When the Hughes press was used the cells were grown on the above medium solidified with 2% (w/v) agar in Roux bottles and incubated at 30° overnight; 15–20 Roux bottles of medium gave 2–5 g. wet weight of cells. After harvesting and washing once in distilled water, the cells were mixed with their own weight of powdered Pyrex glass and transferred to the bacterial press, which had been cooled to −14°, and crushed. After crushing, a smear of the paste was made, fixed and stained with Loeffler's methylene blue and examined microscopically with a 2 mm. objective and a ×10 ocular. The presence of less than ten unbroken cells per field was taken as an indication of a satisfactory crush. The paste of crushed cells and glass was transferred to a tared centrifuge tube, weighed, and 1 ml. of 0·066M phosphate buffer (pH 7·4)/g. of paste added. The glass and cell debris was removed by centrifuging for 5 min. at 9000–10000 rev./min. in an M.S.E. high-speed angle centrifuge. The supernatant consisted of a brownish opalescent liquid in which were suspended pink particles; these two phases were mixed and used together. On a few occasions the supernatant was almost clear and pale yellow and contained no pink particles. Such preparations had no oxidizing capacity.

For 'Mickle' extracts the cells were grown on the liquid medium described above, harvested and washed once in distilled water and then suspended in 0·066M phosphate buffer, pH 7·4, to a concentration of 15–25 mg. dry wt./ml. The thick suspensions were then shaken in the disintegrator with Ballotini beads, grade 12, until stained smears of the suspension showed no more than ten unbroken cells per microscope field; usually 15–30 min. shaking sufficed. The glass beads were filtered off through a sintered-glass funnel, porosity 2, and the supension of crushed cells was centrifuged for 5 min. at 9000–10000 rev./min. The supernatants were similar in appearance to those from block-crushed cells.

The total N of the cell-free extracts was determined by the micro-Kjeldahl procedure of Chibnall, Rees & Williams (1943). The ammonia was distilled off in the apparatus of Markham (1942), and the ammonia trapped in 1 ml. of the boric acid reagent of Conway & O'Malley (1942).

RESULTS

The oxidation of succinate by washed suspensions

Fig. 2 shows a typical progress curve for oxidation of succinate by a washed suspension of the organism. It will be noted that there is a sharp break corresponding to approximately 1·5 moles of oxygen/mole of succinate added. Similar ratios were obtained when the suspensions were allowed to respire in the presence of excess of succinate, and the reaction stopped by the addition of acid and the residual

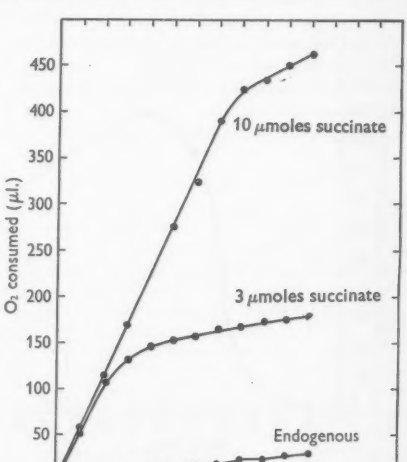


Fig. 2. Oxidation of succinate by washed suspensions grown on succinate. Manometer cups contained: in the side arm 0·5 ml. of bacterial suspension (1·5 mg. dry wt. of cells), in the main cup 0·5 ml. of 0·066M phosphate buffer pH 7·4, substrate as indicated, distilled water to 2·3 ml., in the centre well 0·2 ml. of 20% (w/v) KOH. Temp. 30°.

Table 1. *Consumption of succinate and of oxygen by washed suspensions*

(The total cup contents were: 3·0 ml. containing 0·011 M phosphate buffer pH 7·4 and 0·2 ml. of 20% KOH in the centre well, 0·5 ml. of 50% H_2SO_4 in one side bulb and 0·5 ml. suspension in the second side bulb. Substrate as indicated in the main compartment, and water to volume. Temp. 30°.)

Expt. no.	Succinate added (μmoles)	Suspension (mg. dry wt./cup)	Succinate consumed (μmoles)	Oxygen consumed (μmoles)	Ratio: oxygen / succinate
1	20	1·5	9·07	14·8	1·63
2	30	1·0	13·3	17·3	1·30
3	30	0·95	7·15	9·87	1·38
4	30 + 0·001 M sodium azide	1·5	14·4	19·1	1·32

succinate determined (Table 1). Addition of 0·001 M sodium azide or 0·0001 M 2:4-dinitrophenol as assimilation inhibitors (Clifton, 1937) had no effect on this break in the oxygen uptake curve. Campbell & Stokes (1951) have made similar observations with *Ps. aeruginosa* grown on acetate. As the complete oxidation of succinate to carbon dioxide and water requires 3·5 moles oxygen/mole succinate these results suggested that under our conditions the substrate is incompletely oxidized, with the accumulation of one or more intermediates. On the basis of the observed oxygen:succinate ratio of 1·5, two schemes can be postulated: first, the stepwise oxidation of succinate to acetate with the uptake of 1·5 moles oxygen and the production of 2 moles carbon dioxide according to the following equation:

$$\text{Succinate} + 1{\cdot}5O_2 = \text{acetate} + 2CO_2 + 3H_2O. \quad (1)$$

Secondly, the stepwise oxidation of 1 mole of succinate to 'active' acetate, of a second mole to oxaloacetate, followed by the condensation of these products to give citric acid which would then be oxidized to α-ketoglutarate. The overall equation for this reaction scheme is

$$2\ \text{Succinate} + 3O_2 = \alpha\text{-ketoglutarate} + 3CO_2 + 3H_2O. \quad (2)$$

Measurements of the respiratory quotients of suspensions oxidizing succinate (Table 2) gave values close to 1·2 which does not agree very well with either of these schemes, since the R.Q. for equation (1) is 1·3 and that for equation (2) is 1·0. A small amount of volatile acid, shown by paper chromatography to be acetate, was present in the reaction mixture. Tests for the presence of keto acids were positive. Chromatography of the 2:4-dinitrophenylhydrazones, prepared from cup contents in which succinate had been oxidized, gave three yellow spots, with R_F's 0·162, 0·46 and 0·63, respectively, which were the same as those of the 2:4-dinitrophenylhydrazones of authentic α-ketoglutaric acid and pyruvic acid. Altmann, Crook & Datta (1951), Seligson & Shapiro (1952), Walker *et al.* (1951), and Gordon, Wopart, Burnham & Jones (1951) have also observed that the 2:4-dinitrophenylhydrazone of pyruvic acid often gives two spots, due to *syn-anti* isomerism. Since the 2:4-dinitrophenylhydrazone of oxaloacetate has an R_F similar to that of the α-ketoglutarate derivative, the above procedure was carried out on duplicate samples of supernatants, one of which was heated for 10 min. in a boiling-water bath to decompose any oxaloacetate present and the hydrazones were separated as described above. The chromatograms from these two supernatants were identical, indicating that the slowly moving spot was in all probability the 2:4-dinitrophenylhydrazone of α-ketoglutarate. No keto acids were found in a control without substrates.

Table 2. *Respiratory quotient of succinate oxidation*

(Double side-bulb cups containing 0·5 ml. of suspension (2·0 mg. dry wt./ml.), 0·5 ml. of 0·066 M phosphate buffer pH 7·0, 0·1 ml. of 0·33 M succinate in dangling tube, 0·3 ml. of 5 N-HCl in one side bulb, 0·3 ml. of N-NaOH in second side bulb and water to 2·5 ml. total vol. Temp., 30°.)

No.	Oxygen consumed (μmoles)	CO_2 produced (μmoles)	R.Q.
1	20·7	24·6	1·18
2	19·4	23·9	1·23
3	20·3	24·7	1·21

Table 3 shows the results from a number of experiments in which the amounts of α-ketoglutarate formed from succinate by washed suspensions was estimated by the transaminase method and by the method of Goodwin & Williams (1952). The ratio of moles α-ketoglutarate formed/moles oxygen consumed was found to vary between 0·08 and 0·27, whereas if succinate were oxidized quantitatively to α-ketoglutarate according to equation (2), the ratio should be 0·33. Fig. 3 shows the production of α-ketoglutarate and pyruvate, and the consumption of oxygen plotted against time. The experiment was carried out by using a series of cups and stopping the reaction at suitable time intervals.

Table 3. *Production of α-ketoglutarate from succinate*

(All experiments were performed in double side-bulb cups containing 0·0133 M phosphate buffer pH 7·4, 1·5 mg. dry wt. of cells were tipped in from one side bulb. Acid in the second side bulb and substrate as indicated in the main cup. 0·2 ml. 20% (w/v) KOH in the centre well. Temp. 30°.)

Expt. no.	Succinate added (μmoles)	Time of incubation (min.)	Oxygen consumed (μmoles)	α-Ketoglutarate formed (μmoles)	Ratio: α-ketoglutarate formed/oxygen consumed
4	20	20	12·6	1·70	0·135
	20	30	18·2	2·34	0·128
	20	54	30·9	2·65	0·086
	20	69	32·2	3·11	0·097
	20	134	34·6	2·46	0·071
7	60	25	12·6	3·4	0·27
	60	35	18·1	3·65	0·20
	60	79	32·3	4·30	0·134
	60	99	36·4	4·70	0·129
8	100	15	7·0	1·41	0·20
	100	20	10·8	1·49	0·138
	100	30	15·0	2·38	0·158
	100	40	21·2	3·41	0·161
	100	50	26·6	4·55	0·171

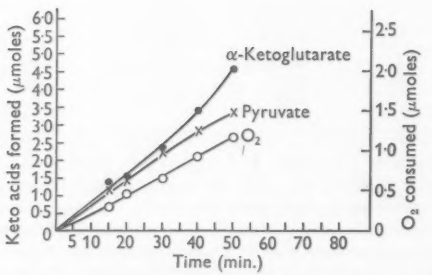


Fig. 3. Oxygen consumption and the production of α-ketoglutarate and pyruvate by washed suspensions oxidizing succinate. Experimental conditions are the same as described in Table 3.

Isolation of α-ketoglutaric acid from the growth medium

Initial attempts to detect α-ketoglutaric acid in the ordinary growth medium were unsuccessful. Lockwood & Stodola (1946), in their work on the production of α-ketoglutarate from glucose, used a medium with a carbon:nitrogen ratio of approx. 100. We therefore modified the usual succinate medium so that it contained 3·6% (w/v) sodium succinate and 0·01% (w/v) ammonium chloride (C:N = 400). The α-ketoglutaric acid was isolated as the 2:4-dinitrophenylhydrazone from the culture medium as follows.

Six penicillin flasks, each containing 300 ml. of the above medium, were inoculated and incubated at 25° for 50 hr. with shaking. At the end of this period the organisms were centrifuged off and the α-ketoglutarate content of a sample of the supernatant was estimated; yield 58 μg./ml. The supernatant was treated with 20 g. of decolorizing charcoal (Hopkin and Williams) and after filtration was concentrated *in vacuo* to 230 ml. The concentrated solution was adjusted to pH 7·0 with conc. HCl and 51 ml. of conc. HCl were added, followed by 300 mg. of 2:4-dinitrophenylhydrazine in 45 ml. of 2N-HCl. The mixture was left at room temperature for 2 hr. and stored overnight in the refrigerator. The crystalline precipitate was filtered off and washed several times with distilled water. The dry precipitate weighed 740 mg. and apparently contained some succinic acid from the growth medium. It was suspended in a little water, transferred to a separating funnel and 30 ml. of ether were added. After shaking for about 1 min., the two layers were separated, and the aqueous phase was washed with successive portions of 20, 10 and 10 ml. ether. The ethereal extracts were combined and extracted with successive portions of 20, 10, 5 and 5 ml. of 15% (w/v) Na_2CO_3. The combined carbonate extracts (40 ml.) were acidified with conc. HCl, when a pale-yellow, crystalline precipitate was formed. This was left in the refrigerator for a few hours, and the precipitate filtered off and washed exhaustively with acidified water to remove any succinic acid. The crystals were dried in a desiccator over $CaCl_2$: yield, 123·7 mg. Paper chromatography of this preparation showed only one spot which corresponded to the 2:4-dinitrophenylhydrazone of α-ketoglutaric acid. The compound was recrystallized from 10 ml. of hot ethanol plus 15 ml. of hot water and the solution allowed to cool. The pale, lemon-yellow, needle-shaped crystals were filtered off, washed with water, and dried in a desiccator over $CaCl_2$: yield, 90 mg. This material had m.p. 219–221° (decomp.) and the mixed melting point with authentic α-ketoglutarate dinitrophenylhydrazone (m.p. 219–220°) (decomp.) was 220–221° (decomp.). (Found: C, 38·7; H, 3·7; N, 15·9; loss of weight on drying *in vacuo* at 50° over P_2O_5 to constant weight, 5·1%. Calc. for $C_{11}H_{10}O_8N_4$, H_2O: C, 38·4; H, 3·5; N, 16·3; H_2O, 5·2%.) Fig. 4 shows the absorption curves of our sample and that of an authentic specimen of 2:4-dinitrophenylhydrazone of α-ketoglutarate, determined in a Beckman spectrophotometer model DU. The $E_{max.}$ for both specimens under these conditions was 1640 at 420–425 mμ.

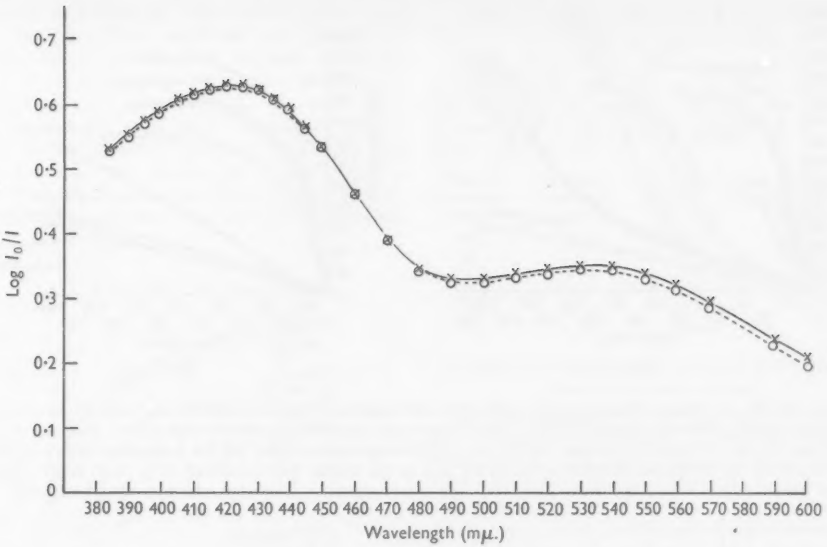


Fig. 4. Absorption spectra of two specimens of the 2:4-dinitrophenylhydrazone of α-ketoglutarate. ×—×, authentic specimen of α-ketoglutaric acid derivative. O- - -O, specimen of α-ketoglutaric acid derivative isolated from the growth medium. The hydrazones were dissolved in 15% (w/v) Na_2CO_3 and an equal volume of 1·5N-NaOH was added. Each solution contained 12·5 μg. of the hydrazone/ml.

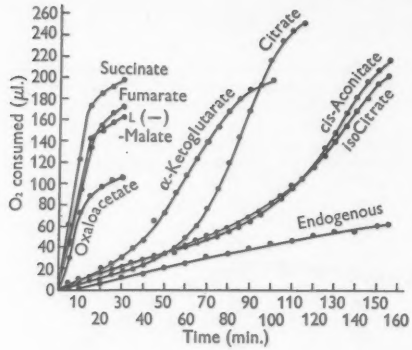
Oxidation of tricarboxylic acid-cycle intermediates by washed suspensions

Since it seemed possible that α-ketoglutarate was formed from succinate by reactions involving the tricarboxylic acid cycle, the oxidation of various cycle intermediates by washed suspensions grown on succinate was next tested. Several of the intermediates were oxidized only after a lag period, suggesting that cells grown upon succinate had to adapt to these substrates. It was found that the lags were very much longer (1–2 hr.) when cells were grown on a liquid medium than when they were grown on the same medium solidified with agar, and consequently all further experiments of this type were carried out with cells which had been grown on solid media dispensed in 100 ml. portions in Roux bottles and incubated at 30° overnight. Fig. 5 (*a*) shows the oxygen uptake plotted against time for the various cycle intermediates by suspensions of cells grown on a succinate medium. From this figure it appears that the cells grown on succinate can oxidize succinate, fumarate, malate and oxaloacetate immediately and linearly, but attack citrate, *cis*-aconitate, *iso*citrate and α-ketoglutarate only after a lag period. Table 4 summarizes a number of experiments in which the oxidation of various cycle intermediates was tested by suspensions of cells grown on different tricarboxylic acid cycle compounds. In these experiments all initial inflexions in the oxygen uptake curves, however slight, have been interpreted as lag periods, on the basis of our experience that the duration and extent of these can fluctuate considerably from experiment to experiment.

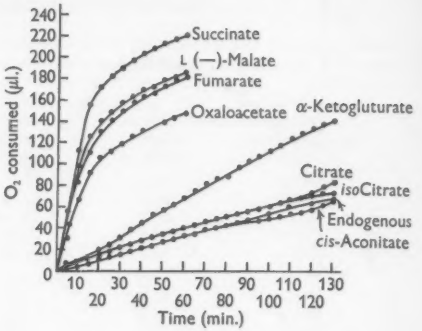
The question arises whether the lag periods observed in the oxidation of some of these substrates by washed suspensions represent enzymic adaptation, i.e. the synthesis of enzymes, and it was thought that an answer might be obtained by use of a specific inhibitor of adaptive-enzyme formation, should one such exist.

Inhibition of adaptation

Irradiation with ultraviolet light seemed to offer possibilities as a specific inhibitor of enzymic adaptation. Swenson & Giese (1950) showed that ultraviolet irradiation inhibited the adaptative formation of galactozymase in yeast. Stanier & Lederberg (1951) have shown that this irradiation inhibits adaptation without affecting oxidative assimilation. Jacob, Torriani & Monod (1951) found that doses which inhibit adaptation in



(a)



(b)

Figs. 5 (*a*) and (*b*). Oxidation of tricarboxylic acid-cycle intermediates by washed suspensions of cells grown on succinate. Oxygen uptake followed manometrically. Manometer cups contained: in the side arm 0·5 ml. bacterial suspension (3·0 mg. dry wt. of cells); in the main cup 0·5 ml. of 0·066 M phosphate buffer pH 7·4, 4 μmoles substrate as indicated (4·15 μmoles of *iso*citrate), distilled water to 2·0 ml.; in the centre well 0·2 ml. of 20% (w/v) KOH. Temp. 30°. Fig. 5 (*a*) = unirradiated suspension. Fig. 5 (*b*) = the same suspension irradiated with u.v. light for 12 min.

Table 4. *Oxidation of tricarboxylic acid cycle intermediates by washed suspensions*

(Cup contents: 2·7 ml. containing 0·5 ml. of 0·066 M phosphate buffer pH 7·4; 3·0 mg. dry wt. of cells in side bulb; and 5 μM substrate, 0·2 ml. 20% KOH in centre well. Temp. 30°.)

	Substrate for oxidation									
Growth substrate	Succinate	Fumarate	Malate	Oxalo-acetate	Pyruvate	Acetate	Citrate	*cis*-Aconitate	*iso*-Citrate	α-Keto-glutarate
Succinate	+	+	+	+	0	0	0	0	0	0
Fumarate	+	+	+	+	.	.	0	.	.	0
Malate	+	+	+	+	.	.	0	.	.	0
Acetate	0	0	.	.	+	+	0	.	.	0
Citrate	0	0	+	.	0	0	+	0	0	0
*iso*Citrate	0	0	+	.	.	.	+	+	+	0
α-Ketoglutarate	0	0	.	.	0	0	0	.	.	+

+ = Substrate oxidized linearly from the moment of tipping.
0 = Substrate not linearly oxidized initially.

bacteria do not affect the ability of the cells to reproduce bacteriophage. Brandt, Freeman & Swenson (1951) found that ultraviolet irradiation inhibited the formation of galactozymase in yeast, but had no effect on the activity of preformed galactozymase, or on glucozymase. Swenson (1952) determined the action spectrum of inhibition of galactozymase formation by ultraviolet irradiation, and found that it had a maximum at 260 mμ.

Figure 5 (*b*) shows the behaviour of the same washed suspensions as used in Fig. 5 (*a*) towards tricarboxylic acid-cycle intermediates after ultraviolet irradiation. Washed cell suspensions were irradiated with a Sterilamp (General Electric Co.). The lamp was housed in a wooden cabinet fitted with a glass window and a pair of holes for manipulation. The cell suspension (5–6 ml.), containing 6 mg. dry wt. cell/ml., was pipetted into a Petri dish (diameter 11 cm.) and shaken by hand for 12 min. at a distance of 33 cm. from the lamp. This treatment was found by experiment to inhibit the adaptive oxidation of benzoate by cells grown upon phenylacetate without affecting in any way the oxidation of phenylacetate. Figs. 6 and 7 show the course of the oxidation of citrate, succinate and α-ketoglutarate by irradiated and non-irradiated suspensions of cells grown on citrate.

In no case did the irradiated suspensions show any augmentation in the rate of oxygen uptake over the initial rate, such as was exhibited by non-

irradiated suspensions. This must mean that ultraviolet irradiation inhibited the process or processes responsible for the increase in oxidation rate in response to substrate, presumably the formation of new enzyme. On the other hand, it can be seen that irradiation affects neither the initial rate of oxygen uptake nor the breaks in the oxygen uptake curves, and Table 5 shows that irradiated cells form α-ketoglutarate from succinate in the same way as do non-irradiated cells.

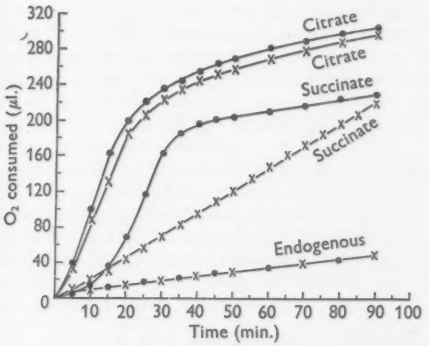


Fig. 6. Oxidation of citrate and succinate by a washed suspension of cells grown on citrate. Experimental conditions as in Fig. 5. ×—×, suspension irradiated with u.v. light; ●—●, non-irradiated control.

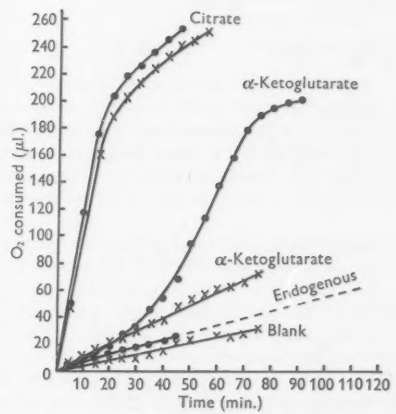


Fig. 7. Oxidation of citrate and α-ketoglutarate by a washed suspension of cells grown on citrate. Experimental conditions as in Figs. 5 and 6. ×—×, suspension irradiated with u.v. light; ●—●, non-irradiated control.

Table 5. *Production of α-ketoglutarate from succinate by irradiated cells*

(Double side-bulb cups containing 0·5 ml. of 0·066 M phosphate buffer pH 7·4, 1·5 mg. dry wt. of cells in one side bulb and acid in the second side bulb. Substrate (60 μmoles) in the main cup, 0·2 ml. 20% (w/v) KOH in centre well. Temp. 30°.)

Time of incubation (min.)	Oxygen consumed (μmoles)	α-Ketoglutarate formed (μmoles)	Ratio: α-ketoglutarate/oxygen consumed
20	9·0	0·96	0·107
30	15·1	2·58	0·171
35	19·6	3·18	0·163
45	24·2	4·25	0·175
50	30·2	3·78	0·125
0	0	0·34	—

Oxidation of tricarboxylic acid cycle intermediates by cell-free preparations

Cell-free preparations made by the two methods were tested for their ability to oxidize the various tricarboxylic acid cycle intermediates. Table 6 shows the results of a number of such experiments with cell-free preparations made from cells grown on either a succinate medium or on a citrate medium. The activities of the extracts, when expressed as $q_{O_2}N$ (μl. O_2/mg. N/hr.) vary considerably from preparation to preparation, and a precise quantitative comparison between the different extracts is therefore impossible. However, it can be seen that the rates of oxidation of succinate by extracts from succinate-grown cells and by extracts from citrate-grown cells are of the same order. The table also gives the activities of the extracts, relative to that with succinate, towards other tricarboxylic acid-cycle intermediates. It will be seen that the growth conditions had no significant effect on the rate of oxidation of these substrates by the extracts. Two 'Mickle' extracts failed to oxidize citrate, and the rate of citrate oxidation by other such extracts was much lower than when extracts prepared with the Hughes press were used. This may be due to inactivation of aconitase during preparation, since tests for the presence of *iso*citric dehydrogenase by the spectrophotometric method of Ochoa (1948) were positive when DL-*iso*citrate was the substrate, but negative when citrate was the substrate. All attempts to prepare extracts which would oxidize acetate failed, whether the Hughes press or the Mickle disintegrator was used, and whether the cells were grown on succinate, citrate or acetate.

The foregoing observations mean that cells, whether grown on succinate or citrate, contain most of the enzymes of the tricarboxylic acid cycle, despite the fact that washed suspensions of the same cells show adaptive phenomena towards some of the tricarboxylic acid-cycle intermediates. Similar observations have recently been published by Barrett, Larson & Kallio (1953).

Table 6. *Oxidations by cell-free extracts*

(Rates, calculated from the O_2 uptake during the first 30 min. after equilibration, are expressed as percentage of the rate on succinate, after subtracting values for blanks. Cup contents were: 2·5 ml. containing 0·5 ml. of 0·066M phosphate buffer pH 7·4, substrate as indicated, 1 μM $MnSO_4$, 2 μM $MgCl_2$, approximately 100 μg. TPN and 200 μg. DPN. 0·5–0·7 μM ATP, 0·2 ml. 20% (w/v) KOH in centre well, and 0·5 ml. extract in the side bulb. Temp. 30°.)

Expt. no.	Substrate (μmoles)	μl. O_2/mg. N/hr.		Rate of oxidation as % of rate with succinate						
		Blank	Succinate	Fumarate	DL-Malate	Oxalo-acetate	Citrate	*cis*-Aconitate	*iso*Citrate	α-Keto-glutarate
				Extracts from cells grown on succinate						
'Mickle' crush										
1	50	.	.	.	23·3	.	0	.	.	17·1
2	50	.	.	.	.		38·0	.	.	.
3	5	.	.	.	.	.	.	.	29·7	.
4	40	.	.	.	.	.	25·8	.	.	.
5	30	5	138	67·0	63·0	.	.	.	27·0	19·6
6	30	.	.	29·6	28·0	.	.	.	17·8	17·5
7	5	7	113	60·6	65·5	17·2	0	27·6	71·5	60·5
8	5	12·9	157	69·5	61·0	23·6	17·0	46·5	45·3	54·5
Block crush										
9	50	13·4	113	.	.	.	60·0	.	.	41·7
10	5	15·9	51	.	.	.	65·0	.	70·0	85·5
				Extracts from cells grown on citrate						
'Mickle' crush										
11	50	.	.	.	92·0	.	6·5	.	.	40·5
12	50	.	.	.	.	.	15·6	.	.	.
13	30	5·7	191	19·2	82·4	.	.	.	94·0	37·6
14	5	9·4	142	40·0	81·0	15·0	6·4	23·4	60·0	40·8

DISCUSSION

We have shown that succinate is oxidized by washed suspensions of a fluorescent *Pseudomonas*, strain KB 1, with an oxygen uptake of approximately 1·5 moles/mole of succinate, compared with 3·5 moles required for the complete oxidation of succinate. This observation led us to look for α-ketoglutarate as one of the end products of this reaction. It is of interest that Campbell & Stokes (1951), working with washed suspensions of *Ps. aeruginosa*, have published graphs which show that succinate was incompletely oxidized and from which it can be calculated that the oxygen uptake was about 1·5 moles oxygen/mole succinate. However, these workers expressed the opinion that the deficit in oxygen consumption was due to assimilation of part of the substrate, despite the fact that none of the inhibitors of oxidative assimilation tested decreased this deficit.

The production of α-ketoglutarate from succinate can be explained in two ways: either it is formed by the reductive carboxylation of succinate, that is to say, a reversal of the α-ketoglutarate oxidase, or it can be produced by a mechanism involving the tricarboxylic acid cycle. These two pathways can be summarized in the following way:

1. (*a*) Succinate + $4H_2O$ → $4CO_2 + 14H^+ + 14e$
   (*b*) $12H^+ + 12e + 3O_2$ → $6H_2O$
   (*c*) $2H^+ + 2e$ + succinate + CO_2 → α-ketoglutarate + H_2O
   2 Succinate + $3O_2$ → α-ketoglutarate + $3CO_2 + 3H_2O$

2. (*a*) Succinate + 1·5 O_2 → acetate + $2CO_2 + H_2O$
   (*b*) Succinate + O_2 → oxaloacetate + H_2O
   (*c*) Acetate + oxaloacetate → citrate
   (*c*) Citrate + 0·5 O_2 → α-ketoglutarate + $CO_2 + H_2O$
   2 Succinate + $3O_2$ → α-ketoglutarate + $3CO_2 + 3H_2O$

With washed suspensions of this organism, the above two equations were never completely satisfied, for not only was the amount of α-ketoglutarate found always less than the theoretical, but there was also some pyruvate produced, and the R.Q. was of the order of 1·2 as opposed to the theoretical value of unity.

Dagley *et al.* (1952) detected α-ketoglutarate when *Vibrio strain* 01 oxidized succinate and suggested that it could be produced either by reductive carboxylation of succinate or via the tricarboxylic acid cycle. Our experiments do not enable us to decide between these two mechanisms, but we have assumed, as a working hypothesis, that the second mechanism is the one which operates in these organisms, and that α-ketoglutarate accumulates as a result of its being formed faster than it is removed. This hypothesis implies that the terminal oxidation of 2-carbon fragments in these organisms proceeds through the tricarboxylic cycle.

Tests of cell-free extracts prepared in different ways showed the following enzyme systems to be present: succinic dehydrogenase, fumarase, malic dehydrogenase, aconitase (in some preparations) *iso*citric dehydrogenase and α-ketoglutaric oxidase. We have not tested for the condensing enzyme, but Ochoa, Stern & Schneider (1951) have reported the extraction of the condensing enzyme from *Ps. fluorescens*. Unlike the cell-free preparations from *Azotobacter vinelandii*, examined by Stone & Wilson (1952), our preparations would not oxidize acetate, even when reinforced with ATP, coenzyme A and catalytic amounts of either fumarate or succinate. Thus, whilst the demonstration that most of the enzymes of the tricarboxylic acid cycle occur in this organism is consistent with the hypothesis that α-ketoglutarate is formed from succinate according to the reactions summarized in scheme (2), it still remains to be shown that the tricarboxylic acid cycle is the only, or the major, mechanism for the complete oxidation of succinate. Quantitative evidence, provided by the ratios of α-ketoglutarate formed/oxygen consumed, show that the formation of this intermediate could account for up to 70% of the oxygen uptake on succinate. Whether the remainder represents the oxygen used for removal of α-ketoglutarate or whether it represents another mechanism, acting simultaneously, for the oxidation of succinate, or some of its oxidation products, remains to be investigated. Attempts to study this question by the use of various inhibitors, such as malonate or arsenite, met with no success. Neither of these had any effect on the oxidation of succinate by washed suspensions possibly because the enzymes concerned were inaccessible; on the other hand, since cell-free extracts did not produce α-ketoglutarate from succinate, the effect of these inhibitors on such preparations was not tested.

When the oxidative capacity of whole cells was examined, we found that cells grown on a succinate medium oxidize citrate, *cis*-aconitate, *iso*citrate and α-ketoglutarate only after a lag period; but when the cells were grown on a citrate medium there was a pronounced lag in the oxidation of α-ketoglutarate. These activities, however, do not reflect the enzymic constitution of the cell, since extracts of cells, no matter how cultured, contained the appropriate

oxidizing enzymes. When suspensions were irradiated with ultraviolet light the augmentation in the rate of oxygen uptake on any of the substrates tested was completely inhibited whilst it did not affect the initial rate of oxygen uptake, nor the location of the breaks in the oxygen uptake curve, nor the production of α-ketoglutarate from succinate. If one assumes that irradiation with ultraviolet light inhibits specifically the synthesis of new enzyme, then the above results can only mean that the lag periods are manifestations of adaptive enzyme formation.

Assuming this to be true, what is the nature of the enzymes synthesized? There are two possible explanations. First, that growth on a given substrate *A*, which is oxidized via intermediates, *B*, *C*, *D*, etc., will lead to the formation of a certain quantity of enzymes, *Eb*, *Ec*, *Ed*, etc., to deal with these intermediates. Incubation of washed suspensions with relatively high concentrations of one of these intermediates might then require the formation of further enzyme for its oxidation at maximum rate. Against this hypothesis are the following considerations. (1) The difference in behaviour of cells grown on succinate towards fumarate, malate and oxaloacetate on the one hand, and citrate, *iso*citrate and α-ketoglutarate on the other hand. That is to say, growth on succinate appears to lead to the synthesis of sufficient fumarase, malic dehydrogenase and oxaloacetate decarboxylase to deal with high concentrations of these substrates supplied in the medium, but not to the synthesis of enough aconitase, *iso*citric dehydrogenase or α-ketoglutarate oxidase to deal with equivalent amounts of citrate, *iso*citrate or α-ketoglutarate. (2) Cell-free extracts from succinate-grown cells oxidize *iso*citrate or α-ketoglutarate at rates comparable with those of citrate-grown extract, although the washed suspensions of succinate-grown cells show a complete lag of 30 min. on citrate or *iso*citrate.

The second possibility is that the adaptation involved in these experiments is not concerned with the actual oxidative steps, but with making the substrates accessible to the enzyme, either across the cell membrane or to some special site within the cell. On this hypothesis the adaptive process would consist of the formation of the particular enzyme necessary for the movement of substrate.

Campbell & Stokes (1951) observed that washed suspensions of *Ps. aeruginosa*, prepared from cultures grown on acetate, only oxidized certain of the tricarboxylic acid-cycle intermediates after a lag period, whereas dried preparations made from cells grown on the same medium oxidized these same intermediates. They commented as follows: 'Apparently the cell membrane of *Ps. aeruginosa* was initially impermeable to the compounds mentioned, and the period of adaptation encountered with resting cells was the time necessary for the elaboration of the system for transferring the substrates across the membrane'. Stone & Wilson (1952) observed a similar phenomenon in *Az. vinelandii* and remarked: 'Results from simultaneous adaptation experiments must be interpreted with considerable reservation, unless the factor of permeability can be eliminated. Unfortunately, this is seldom possible, since "permeability" here implies not only ability to penetrate the cell wall, but also to get to the enzyme surface at which the reaction occurs.' Neither of these groups of workers, however, investigated whether the lag periods they observed involved the formation of new enzyme.

Gary & Bard (1952) observed lag periods in the oxidation of gluconate and pentose by resting cells of *B. subtilis* grown on a simple medium containing glucose, and interpreted these as 'corresponding to the time required for the breakdown of a permeability barrier, or its equivalent, the synthesis or activation of the respective kinases'. This statement was not supported by any direct evidence on the nature of the lag. Barrett *et al.* (1953) studied the effect of various inhibitors of enzymic adaptation on the lag period which occurs when washed suspensions of *Ps. fluorescens*, grown on fumarate, are given citrate to oxidize. They found that the inhibitors tested prolonged the lag period indefinitely. They also compared the rates of citrate oxidation by extracts of cells grown on fumarate and by extracts of cells adapted to citrate, and found no significant difference in the two extracts. They commented thus: 'It may be that some type of transport or carrier system is being synthesized by fumarate-grown cells and its completion would enable citrate to be carried across the osmotic barrier and perhaps oriented on the enzyme surface.' A 'carrier' concept has also been discussed by Doudoroff (1951) and by Osterhout (1952), but not in connexion with adaptive enzyme formation.

In view of the above observations by ourselves, and by the other workers cited, the interpretation of simultaneous adaptation experiments must be modified to include the possibility of a 'permeability' adaptation. Stanier (1950) has pointed out that the validity of the simultaneous adaptation technique rests on the assumption that the cell is permeable to all substrates tested, but it has so far been insufficiently realized that the adaptation itself may be concerned with making the substrate accessible to the enzyme system.

SUMMARY

1. A strain of the *Pseudomonas fluorescens* species group (KB 1), isolated from soil, was found to oxidize succinate incompletely, with the uptake

of approximately 1·5 moles oxygen/mole succinate and with the accumulation of α-ketoglutarate and pyruvate. The yield of α-ketoglutarate formed from succinate was variable, but could account for up to 70% of the oxygen consumed.

2. α-Ketoglutaric acid as the 2:4-dinitrophenylhydrazone was isolated from a growth medium containing succinate as sole source of carbon.

3. Washed suspensions of cells grown with succinate as the sole source of carbon oxidized citrate, *iso*citrate, *cis*-aconitate and α-ketoglutarate after a lag period. These adaptations were inhibited by irradiating the suspensions with ultraviolet light.

4. Washed suspensions of cells grown with citrate as the sole source of carbon-oxidized succinate and α-ketoglutarate only after a lag period and irradiation of the suspension with ultraviolet light inhibited these adaptations.

5. Cell-free extracts of cells grown on a medium containing either succinate or citrate were found to contain the following enzymes: succinic dehydrogenase, fumarase, malic dehydrogenase, oxaloacetic decarboxylase, aconitase, *iso*citric dehydrogenase and α-ketoglutaric oxidase.

6. The role of the tricarboxylic acid cycle in the oxidation of succinate by this organism is discussed.

7. The use of the technique of simultaneous adaptation for the study of the tricarboxylic acid cycle in micro-organisms is discussed.

It gives us great pleasure to thank Dr S. R. Elsden for his constant advice and encouragement and much valuable criticism during the course of this work.

We wish to thank Prof. H. A. Krebs, F.R.S., for gifts of oxaloacetic acid, trichloromethylparaconic acid and for specimens of the 2:4-dinitrophenylhydrazones of α-ketoglutaric and pyruvic acid; and Dr P. W. Brian of Imperial Chemical Industries Ltd., for the micro-analysis. One of us (M.K.) wishes to thank the Agricultural Research Council for a training grant.

REFERENCES

Altmann, S. M., Crook, E. M. & Datta, S. P. (1951). *Biochem. J.* **49**, lxiii.
Barker, H. A. & Beck, J. V. (1942). *J. Bact.* **43**, 291.
Barrett, J., Larson, A. & Kallio, R. (1953). *J. Bact.* **65**, 187.
Bergey, D. H. (1948). *Manual of Determinative Bacteriology*, 6th ed. London: Ballière, Tindall and Cox.
Brandt, C. L., Freeman, P. J. & Swenson, P. A. (1951). *Science*, **113**, 383.
Campbell, J. J. R. & Stokes, N. F. (1951). *J. biol. Chem.* **190**, 853.
Cavallini, D., Frontali, N. & Toschi, G. (1949). *Nature, Lond.*, **163**, 568.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 355.
Clifton, C. E. (1937). *Enzymologia*, **4**, 246.
Clutterbuck, P. W. (1927). *Biochem. J.* **21**, 513.
Conway, E. J. & O'Malley, E. (1942). *Biochem. J.* **36**, 655.
Dagley, S., Fewster, M. E. & Happold, F. C. (1952). *J. Bact.* **63**, 327.
Dickens, F. & Šimer, F. (1930). *Biochem. J.* **24**, 905.
Doudoroff, M. (1951). *Phosphorus Metabolism*, ed. by McElray, W. D. & Glass, B. Baltimore: Johns Hopkins Press.
Elsden, S. R. & Lewis, D. (1953). *Biochem. J.* **55**, 183.
Gale, E. F. & Stephenson, M. (1938). *Biochem. J.* **32**, 392.
Gale, E. F. & Stephenson, M. (1939). *Biochem. J.* **33**, 1245.
Gary, N. D. & Bard, R. L. (1952). *J. Bact.* **64**, 501.
Goodwin, T. W. & Williams, G. R. (1952). *Biochem. J.* **51**, 708.
Gordon, B. E., Wopart, F. jun., Burnham, N. D. & Jones, C. C. jun. (1951). *Analyt. Chem.* **23**, 1754.
Hockenhull, D. J. D., Wilkin, G. D. & Winder, F. G. (1951). *Nature, Lond.*, **168**, 1043.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Hunter, G. J. E. (1952). *Proc. Univ. Otago med. Sch.* **30**, 10.
Jacob, F., Torriani, A. M. & Monod, J. (1951). *C.R. Acad. Sci., Paris*, **223**, 1230.
Kogut, M. (1952*a*). *J. gen. Microbiol.* **7**, v.
Kogut, M. (1952*b*). *2nd Int. Congr. Biochem. Abstr.* p. 87.
Krebs, H. A. (1937). *Biochem. J.* **31**, 2095.
Krebs, H. A. (1950). *Biochem. J.* **47**, 605.
Lepage, G. A. & Mueller, G. C. (1949). *J. biol. Chem.* **150**, 975.
Lockwood, L. B. & Stodola, F. H. (1946). *J. biol. Chem.* **164**, 81.
Lohmann, K. (1931). *Biochem. Z.* **233**, 460.
Malachowski, R. & Maslowski, M. (1928). *Ber. dtsch. chem. Ges.* **61** B, 2531.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Mickle, H. (1948). *J. R. micr. Soc.* **68**, 10.
Needham, D. M. (1942). *Biochem. J.* **36**, 113.
Ochoa, S. (1948). *J. biol. Chem.* **174**, 133.
Ochoa, S., Stern, J. R. & Schneider, M. C. (1951). *J. biol. Chem.* **193**, 691.
Osterhout, W. J. V. (1952). *J. gen. Physiol.* **35**, 579.
Racker, E. (1950). *J. biol. Chem.* **184**, 313.
Seligson, D. & Shapiro, B. (1952). *Analyt. chem.* **24**, 754.
Stamp, Lord (1947). *J. gen. Microbiol.* **1**, 251.
Stanier, R. Y. (1947). *J. Bact.* **54**, 339.
Stanier, R. Y. (1950). *Bact. Rev.* **14**, 179.
Stanier, R. Y. & Lederberg, J. (1951). Personal communication.
Stone, R. W. & Wilson, P. W. (1952). *J. Bact.* **63**, 605.
Swenson, P. A. (1952). *Proc. nat. Acad. Sci., Wash.*, **36**, 699.
Swenson, P. A. & Giese, A. C. (1950). *J. cell comp. Physiol.* **36**, 369.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1949). *Manometric Techniques and Tissue Metabolism*, 2nd ed. Minneapolis, U.S.A.: Burgess Publishing Co.
Walker, T. K., Hall, A. N. & Hopton, J. W. (1951). *Nature, Lond.*, **168**, 1042.
Wooldridge, W. R. Knox, R. & Glass, V. (1936). *Biochem. J.* **30**, 926.

Observations on Brain Phosphatases

By J. J. GORDON
Biochemical and Endocrinological Research Department, Barrow Gurney Hospital, Barrow Gurney, near Bristol

(*Received* 6 *March* 1953)

The presence of an 'ordinary' phosphatase (i.e. phosphomonoesterase) in brain was reported by Vondracek (1927). Activities, however, were four to fifteen times lower than in the kidney (King, 1931; Macfarlane, Patterson & Robison, 1934). Giri & Datta (1936) showed that acetone-dried powders prepared from brain can hydrolyse both β-glycerophosphate and hexose diphosphate at the optimum pH values of 5·0 and 9·4. Brain tissue also hydrolyses adenosine 5′-phosphate (Reis, 1937), adenosine triphosphate (ATP) (Dubois & Potter, 1943), and inorganic pyrophosphate (Gordon, 1950). Distribution of phosphatases in different regions of ox and human brain has been studied chemically by Fleischhacker & Yates (1936) and Fleischhacker (1938), and certain variations in activity were noted. Other investigations of phosphatase activity in the brain and nervous system have been carried out by the use of histological techniques (Landow, Kabat & Newman, 1942; Wolf, Kabat & Newman, 1943; Naidoo & Pratt, 1951, 1952).

The present investigation was undertaken to examine more extensively the distribution of phosphomonoesterase and pyrophosphatase (PPase) in the brain, and to obtain values for normal human brains for comparison with brains obtained *post mortem* from cases of psychosis. Some evidence has been presented by Binkley & Olson (1950) that inorganic pyrophosphate and ATP may be acted upon by the same enzyme system in brain, and since this point was of possible importance in interpreting the results of PPase measurements, experiments were carried out on rat-brain preparations to compare the properties of adenosine triphosphatase (ATPase) and PPase. An account of this work is included in the present paper. These experiments support the conclusion reached by Gore (1952), using guinea pig brain, that ATPase and PPase are separate systems.

METHODS

Enzyme preparations. Aqueous dispersions of brain, and suspensions made from acetone-dried powders of brain, were used as sources of enzyme.

Acetone-dried powders were prepared from whole rat brains. The brains were ground with ice-cold acetone (2–3 ml.) in a Potter-Elvehjem (1936) homogenizer. The solid matter was then washed successively with ice-cold acetone, aqueous acetone, and ether, and finally dried, according to the procedure previously employed (Gordon, 1950). When required for use, the dry powder (50–100 mg.) was ground with glycerol (2·5–5·0 ml.) to a paste and then made into a suspension in water.

Aqueous dispersions of rat-brain tissue were made by grinding whole rat brains with ice-cold 0·9 % NaCl (2–3 ml.). For phosphomonoesterase measurement the dispersion was diluted to 150 mg./ml. wet weight and dialysed in a Visking cellophan tube (¼ in. diameter) against running water for 16 hr., to remove inorganic phosphate. For PPase and ATPase measurements the dispersions were diluted to 10–15 mg./ml., but were not dialysed.

Normal human brains were obtained from the Bristol General Hospital, from cases where a post-mortem examination was conducted within 24 hr. of death. Such brains were packed in cooled porcelain containers, sealed over with cellophan, transported to the laboratory and dissected as soon as possible afterwards. Small quantities (up to 1 g. where possible) of tissue from various regions were dissected out and transferred to tared stoppered weighing bottles, which were then re-weighed. The tissue was transferred with the minimum amount of 0·9 % NaCl to suitable tubes, ground as usual, and made to the appropriate concentrations; the dispersions which were required for phosphomonoesterase measurement were dialysed, as above.

Sheep brains, obtained from the abattoir, were packed on ice, transported to the laboratory and dissected and ground in the same manner as human brain.

Enzyme assay. Estimations of phosphomonoesterase and PPase were carried out at 37° in the same general manner as previously described (Gordon, 1950) with certain variations.

PPase activity was measured at pH 7·4 in veronal buffer medium. Tissue dispersions were used at a final concentration of 2·5 mg./ml. wet weight, a Mg^{2+} concentration of 0·008 M and a substrate concentration of 0·001 M. Acetone-powder preparations were used as before (Gordon, 1950), but with a substrate concentration of 0·001 M. In the experiments on sheep and human brain, cysteine (0·01 M) was included as an activator.

For phosphomonoesterase measurements the tissue dispersions were used at a final concentration of 18 mg./ml. wet weight. Sodium β-glycerophosphate (0·0075 M) was used as substrate. Acid phosphatase was measured in presence of acetate buffer (pH 5·0) using a 2 hr. incubation period. Alkaline phosphatase was measured in presence of veronal buffer (pH 9·4) using a 1 hr. incubation period.

ATPase activity was estimated at pH 7·4, using veronal buffer. Brain dispersions were used at a final concentration of 2·5–5·0 mg./ml. wet weight. The ATP concentration was 0·003 M, and Mg^{2+} (0·002 M) was also present. An incubation period of 15 min. was allowed. Acetone-dried powder preparations were used at a final concentration of 2·5–5·0 mg./ml., with a Mg^{2+} concentration of 0·01 M.

Table 1. *Effects of magnesium and calcium ions on adenosine triphosphatase and pyrophosphatase of rat brain*

Ion	Concentration (M)	Activity (mM substrate/30 min./100 g.) PPase (dispersion)	ATPase (dispersion)	ATPase (acetone prep.)
None		0	10·8	0
Ca^{2+}	$1·5 \times 10^{-4}$	—	20·5	0
Ca^{2+}	10^{-3}	0	22·0	0
Mg^{2+}	3×10^{-4}	—	28·3	—
Mg^{2+}	2×10^{-3}	23·5	46·2	—
Mg^{2+}	4×10^{-3}	32·0	46·0	3·8
Mg^{2+}	8×10^{-3}	34·5	—	—
Mg^{2+}	10^{-2}	—	—	4·5
Mg^{2+}	$1·6 \times 10^{-2}$	34·5	—	—

All results are expressed as mM substrate/30 min./100 g. wet-weight tissue. It was confirmed by independent experiments that the progress of the phosphomonoesterase reaction at pH 5·0 and 9·4 was linear over the limits of the assay times employed. In the case of ATPase it was assumed that only one orthophosphate group was liberated during the short period of incubation employed.

ATP (barium salt), obtained from Boots Pure Drug Co., was washed with acetate buffer (pH 4·0, 0·1 M) as recommended by Kornberg (1950), and converted into the sodium salt as described by Bailey (1942). The concentration was checked by a 7 min. hydrolysis test.

RESULTS

Adenosine triphosphatase activity

The present findings for rat brain agree broadly with those of Gore (1952) for guinea-pig brain. Activity was maximum at pH 7·4 over a concentration range of $3–5 \times 10^{-3}$ M. Hydrolysis was linear up to 23 % decomposition. It has previously been observed (Feldberg & Mann, 1945; Meyerhof & Wilson, 1948; Gore, 1952) that ATPase activity of brain tissue is destroyed by acetone drying. In the present study, acetone powders showed much lower activity than tissue dispersions (approximately 8–16 %, referred to weight of original tissue). The activity was still measurable, however, and a number of experiments recorded later in the paper were performed on suspensions of acetone-dried powders.

Effects of magnesium and calcium ions on pyrophosphatase and adenosine triphosphatase

Brain PPase, in common with the PPases from various other sources, is activated by Mg^{2+} and inhibited by Ca^{2+} (Gordon, 1950). ATPase in brain has previously been reported to be activated by Ca^{2+} (Dubois & Potter, 1943; Feldberg & Mann, 1945; Epelbaum, Sheves & Kobylin, 1949), but Gore (1952) was unable to demonstrate this. It can be seen from Table 1 that dispersions of fresh brain showed appreciable ATPase activity even in absence of an activating ion; presence of Ca^{2+} increased this activity by more than 100 %, but a much greater effect was shown by Mg^{2+} at an optimum concentration of 0·002 M. Acetone-powder preparations showed some ATPase activity, at a greatly reduced level, only in presence of Mg^{2+} ions; Ca^{2+} ions were ineffective. In contrast, PPase in fresh tissue dispersions was not activated by Ca^{2+} but was activated best by Mg^{2+} at 0·008 M, compared with 0·015 M previously found with acetone preparations (Gordon, 1950). Gore (1952) found that ATPase in guinea pig brain dispersions was inhibited by Ca^{2+} at 6×10^{-4} M. This difference, however, is probably accounted for by the different conditions employed. Gore's assays were carried out in presence of glycylglycine buffer, and it was noted that under these conditions the ATPase activity may be three times as high as with veronal buffer (Dubois & Potter (1943), who demonstrated an activation by Ca^{2+}, used veronal buffer).

Table 2. *Effects of dialysis and cysteine on pyrophosphatase and adenosine triphosphatase activities of rat brain*

	% change PPase	ATPase
Activity lost on storage at 0°	15·5	25·7
Activity lost on dialysis	87·2	51·3
Activation of fresh enzyme by 0·01 M cysteine	16·5	21·7
Activity restored to dialysed enzyme by 0·01 M cysteine	42·0	55·5

Effects of dialysis

When brain dispersions (10 mg./ml.) were dialysed against a large volume of water at 0° for 18 hr., considerable loss of PPase activity occurred, while storage of the preparation at 0° for the same time resulted in relatively only slight loss (Table 2). This lability on dialysis does not appear to be due to loss of a diffusible coenzyme, since attempts to demonstrate reactivating properties in the dialysate have failed. It is of interest, however, that cysteine, which causes moderate activation of the undialysed enzyme, also restores a large proportion of the lost activity. ATPase on dialysis also showed loss of

activity, which however was much less marked; in this case, only slight restoration of activity was brought about by cysteine, which also caused slight activation of the undialysed enzyme.

Fractionation of pyrophosphatase and adenosine triphosphatase

Experiments were attempted to determine whether a partial separation of ATPase and PPase could be achieved. 10 ml. portions of a fresh rat-brain dispersion (100 mg./ml.) were first treated with saturated ammonium sulphate solution to bring the mixture to a definite saturation level. After standing for 30 min. at 0°, the precipitates were centrifuged and resuspended in water (8 ml.) and, after diluting suitably, the activities were determined as usual. Control assays on the original dispersion were also carried out. It was confirmed by experiment that ammonium sulphate at 10 mg./ml. concentration did not directly affect the activities. Results shown in Table 3 indicate that PPase and ATPase both tend to concentrate mainly in the 30–40 % saturation fractions, but whereas only 20 % of the PPase activity is found in the 40 %-fraction, ATPase is found to the extent of 40 % in the 20 %-fraction and 64 % in the 30 %-fraction. A difference between ATPase and PPase was also seen when ethanol fractionation was tried. Though maximum concentration was found in both cases at 40 % saturation, 38 % of the PPase and only 21 % of the ATPase activity were found in this fraction.

Table 3. *Fractionation of pyrophosphatase and adenosine triphosphatase activities of rat brain by ammonium sulphate or ethanol*

(10 ml. of 100 mg./ml. dispersion treated with different concentrations of $(NH_4)_2SO_4$ or ethanol and centrifuged; precipitates suspended in 8 ml. water.)

Reagent	Concentration (%, w/v)	% Activity found in precipitate	
		PPase	ATPase
$(NH_4)_2SO_4$	10	8·4	21·0
$(NH_4)_2SO_4$	20	9·8	40·0
$(NH_4)_2SO_4$	30	12·6	64·0
$(NH_4)_2SO_4$	40	20·2	71·0
Ethanol	20	1·7	3·3
Ethanol	40	38·2	21·1
Ethanol	60	38·2	23·3
Ethanol	80	41·2	23·3

Effects of inhibitors

Gore (1952) found that ATPase and PPase of guinea pig brain differed in their behaviour towards certain inhibitors. In the present study, experiments were carried out on rat brain, using aqueous dispersions and acetone-dried preparations, to determine whether there are differences in sensitivity between the two enzymes towards various compounds. The results reported in Table 4 would appear to give some indication that such differences may exist. Zinc, iron or copper sulphate, mercuric chloride, sodium fluoride, sodium cyanide and mapharside (*m*-amino-*p*-hydroxyphenylarsenoxide) all brought about strong inhibition of PPase activity in the aqueous dispersions, at concentrations which caused only slight or moderate inhibition of ATPase. The differences were less marked in some instances (especially zinc sulphate and sodium fluoride) when acetone preparations were used for the activity measurements. Alloxan and

Table 4. *Inhibition of rat brain pyrophosphatase and adenosine triphosphatase by various compounds*

Inhibitor	Concentration (M)	Inhibition (%)			
		Dispersion		Acetone preparation	
		PPase	ATPase	PPase	ATPase
$ZnSO_4$	4×10^{-5}	98	0	97	45
$ZnSO_4$	4×10^{-6}	82	0	35	8
$FeSO_4$	4×10^{-4}	96	28	100	43
$FeSO_4$	4×10^{-5}	93	21	87	41
$CuSO_4$	5×10^{-5}	100	51	100	52
$CuSO_4$	2×10^{-5}	93	0	100	17
$HgCl_2$	2×10^{-5}	97	54	97	52
$HgCl_2$	$2{\cdot}5 \times 10^{-6}$	91	6	23	0
$CaCl_2$	$1{\cdot}5 \times 10^{-3}$	99	10	87	79
$CaCl_2$	$2{\cdot}5 \times 10^{-4}$	75	16	66	54
NaF	1×10^{-4}	85	6	93	47
NaF	5×10^{-5}	71	0	76	16
NaCN	2×10^{-2}	31	0	43	0
Mapharside	2×10^{-5}	78	22	77	0
Mapharside	1×10^{-6}	25	6	30	0
Alloxan	5×10^{-3}	99	48	96	63
Alloxan	5×10^{-4}	71	23	80	44
Formaldehyde	$2{\cdot}5 \times 10^{-2}$	87	47	94	89
Formaldehyde	$2{\cdot}5 \times 10^{-4}$	23	0	36	16
Suramin	4×10^{-4}	84	62	75	54

formaldehyde inhibited PPase more strongly than ATPase, but the differences were not as great as with most of the other substances. Suramin inhibited both enzymes to a comparable extent. Calcium chloride inhibited PPase; it did not greatly inhibit ATPase in the aqueous dispersions, but caused marked inhibition of ATPase in the acetone-powder preparations. A strict comparison between the behaviour of the dispersions and acetone preparations is not really possible, since both preparations contained much inert material, and a large proportion of the ATPase in the acetone preparations would most likely have been destroyed by the acetone treatment. It is of interest, however, that Gore (1952) also found that sodium fluoride, copper sulphate and alloxan exerted a relatively greater inhibitory effect on PPase than on ATPase.

Distribution of phosphatases in brain

Tables 5 and 6 show representative results for the phosphomonoesterase and PPase levels in different regions of sheep and human brain. It has not been possible, so far, to examine a sufficient number of human brains to be able to deduce normal levels for comparison with abnormal brains, as there were, in some cases, rather large differences between individual brains. Relative variations throughout the same brain, however, showed a fairly consistent pattern, and the general trend can be seen in the examples quoted.

In sheep brain, the highest values for phosphomonoesterase were found in the occipital cortex, and for PPase in the superior colliculus. The corpus callosum, a conducting tissue, showed much lower activities than the other regions for acid and alkaline phosphatase, but not for PPase. Values for the medulla were in no case significantly lower than in the nucleated regions. These results do not offer evidence of a simple relation between enzyme activity and structure or function.

With human brains the pattern was different; some interesting variations in activity of PPase may be noted. High values occurred in the caudate nuclei, the thalamus, the globus pallidus, the frontal cortex, the putamen, the hypothalamus and the floor of the fourth ventricle, all of which are nucleated regions of high functional significance. Much lower values were found in the medulla (olive)

Table 5. *Distribution of phosphatases in sheep brain*

(Activities expressed as mM substrate/30 min./100 g. tissue.)

	Alkaline phosphatase		Acid phosphatase		PPase	
Region	Brain 1	Brain 2	Brain 1	Brain 2	Brain 1	Brain 2
Caudate nuclei	2·30	1·93	0·36	0·73	28·6	29·6
Superior colliculus	2·45	2·21	0·39	0·54	41·6	43·2
Medulla	1·94	2·46	0·26	0·49	31·1	28·7
Thalamus	1·94	2·32	0·28	0·39	29·1	29·1
Occipital cortex	2·95	2·84	0·44	0·81	32·2	34·1
Corpus callosum	0·61	1·03	0·16	0·07	34·2	29·3
Hypothalamus	2·19	1·98	0·29	0·49	38·6	41·0

Table 6. *Distribution of phosphatases in normal human brain*

(Activities expressed as mM substrate/30 min./100 g. tissue.)

	Alkaline phosphatase				Acid phosphatase				PPase			
Region	Brain 1	Brain 2	Brain 3	Brain 4	Brain 1	Brain 2	Brain 3	Brain 4	Brain 1	Brain 2	Brain 3	Brain 4
Caudate nuclei	1·65	0·82	—	—	0·52	0·72	—	—	26·5	20·6	—	—
Medulla (pyramids)	1·05	0·69	—	—	0·42	0·25	—	—	13·8	11·5	—	—
Medulla (olive)	1·19	0·64	—	—	0·34	0·41	—	—	17·8	18·1	—	—
Thalamus	1·77	0·82	—	—	0·55	0·75	—	—	30·1	30·0	—	—
Globus pallidus	1·34	1·15	—	—	0·41	0·67	—	—	28·1	26·6	—	—
Frontal cortex	2·06	1·34	—	—	0·55	0·72	—	—	28·3	29·4	—	—
Prefrontal cortex	—	—	1·77	1·46	—	—	0·67	0·66	—	—	21·3	20·1
Motor cortex	—	—	1·34	1·17	—	—	0·64	0·61	—	—	23·0	17·3
Sensory cortex	—	—	1·50	1·50	—	—	0·71	0·62	—	—	27·0	20·6
Temporal cortex	—	—	1·77	1·80	—	—	0·68	0·69	—	—	22·9	20·0
Palaeo-cerebellar cortex	—	—	1·45	1·20	—	—	0·86	0·73	—	—	36·5	48·6
Neocerebellar cortex	—	—	1·51	1·24	—	—	0·87	0·69	—	—	42·0	50·7
Hippocampus	—	—	1·32	1·22	—	—	0·78	0·63	—	—	23·9	23·7
Corpus callosum	1·05	0·43	—	—	0·26	0·37	—	—	17·3	16·0	—	—
Putamen	1·96	1·17	—	—	0·57	0·67	—	—	33·5	28·1	—	—
Hypothalamus	1·72	0·88	—	—	0·58	0·80	—	—	32·3	29·4	—	—
Fornix	0·60	0·54	—	—	0·28	0·27	—	—	14·5	13·4	—	—
Floor of 4th ventricle	1·58	0·60	—	—	0·55	0·44	—	—	29·0	24·2	—	—

region, a nucleated region with much conducting tissue, and in the corpus callosum, a definite conducting region. Lowest values were found in the medulla (pyramid) and the fornix, which are also conducting regions. There were no marked or significant differences between the different areas of cerebral cortex, but it is of interest that values in the cerebellar cortex were markedly higher than in the cerebral cortex.

Alkaline phosphatase of human brain also appears to be lowest in the conducting regions. There was appreciable variation between the nucleated regions, however; the floor of the fourth ventricle (brain 2, Table 5) was not much more active than the fornix. The caudate nuclei, the thalamus and the hypothalamus gave intermediate values, while the highest values were given by the globus pallidus, the prefrontal cortex and the putamen.

Acid phosphatase also showed lowest values in the conducting regions, but did not show some variation between nucleated areas as alkaline phosphatase did.

DISCUSSION

It will be noted that, though there are certain similarities between the properties of ATPase and PPase (notably in regard to their activation by cysteine), in other respects there are differences. The partial separation of the two activities achieved by precipitation with ammonium sulphate or ethanol is in agreement with Gore's (1952) success in separating the activities by extraction procedures. Some caution is necessary in drawing conclusions from the inhibition experiments, since crude enzyme preparations only were used, and the results may have been influenced by inert material (especially protein). There appears to be some likelihood that PPase is more readily inhibited than ATPase by most of the compounds tested, though it would be necessary to study this problem using purified enzyme preparations, before any real conclusions could be drawn from such differences. The observations reported, however, tend to support Gore's (1952) conclusions regarding the non-identity of the PPase and ATPase systems in brain.

The importance of enzyme distribution studies in brain has been emphasized by Ashby (1951) who reported wide variations in carbonic anhydrase activity between different regions. In the present study, the PPase levels show a distribution pattern which is related to the histological features of the different regions. Highly nucleated regions in general are much more active than the conducting regions, and this factor appears to account mainly for the relative differences observed. At the same time, the PPase values for cerebellar cortex are considerably higher than for cerebral cortex; it does not seem likely that this phenomenon could be accounted for by the small histological difference between these types of tissue. The possible significance of the PPase distribution pattern is not apparent at present, since little is known about the metabolic role of PPase.

The same tendency for higher values to occur in nucleated regions, and lower values in conducting regions, is seen in the distribution of acid and alkaline phosphatase activity. It is of interest in this connexion that Richter & Hullin (1951) found a strong alkaline phosphatase reaction in cell nuclei isolated from cerebral cortex. Naidoo & Pratt (1951) demonstrated histologically that acid phosphatase activity, with glycerophosphate as substrate, was localized mainly in the neuronal cytoplasm; the cerebral cortex showed cells clearly defined by the staining. It seems probable on the whole that a better idea of the role of the phosphomonoesterase in brain will be obtained by studying the variations between different cellular layers, rather than between whole regions; some interesting observations of this type have already been made by Scharrer & Sinden (1949).

SUMMARY

1. The pyrophosphatase and adenosine triphosphatase activities of rat-brain dispersions may be partially separated by fractionation with ammonium sulphate or ethanol. Both enzymes lose activity on dialysis, and both are activated by cysteine.

2. Pyrophosphatase activity in crude preparations obtained from rat brains is strongly inhibited by a number of substances (metal salts, mapharside, formaldehyde and suramin), most of which exert relatively little effect on adenosine triphosphatase activity.

3. The distribution of phosphomonoesterase and pyrophosphatase activities in different regions of human and sheep brain has been studied. Activities tend to be high in the nucleated regions, and low in conducting regions.

I wish to express my thanks to Mr R. J. Everson for valuable technical assistance; to Dr G. S. Andrews, Pathologist at the Bristol General Hospital, for arranging the supply of normal brains; and to Dr K. W. Aron for carrying out the dissections of human and sheep brains.

REFERENCES

Ashby, W. (1951). *J. nerv. ment. Dis.* **114**, 391.
Bailey, K. (1942). *Biochem. J.* **36**, 121.
Binkley, F. & Olson, C. K. (1950). *J. biol. Chem.* **186**, 725.
Dubois, K. P. & Potter, V. R. (1943). *J. biol. Chem.* **150**, 185.
Epelbaum, S. E., Sheves, G. & Kobylin, A. A. (1949). *Biochemistry, Leningr.*, **14**, 107.
Feldberg, W. & Mann, T. (1945). *J. Physiol.* **103**, 28*P*.
Fleischhacker, H. H. (1938). *J. ment. Sci.* **84**, 947.
Fleischhacker, H. H. & Yates, E. D. (1936). *Chem. and Ind.* **14**, 943.
Giri, K. V. & Datta, N. C. (1936). *Biochem. J.* **30**, 1089.
Gordon, J. J. (1950). *Biochem. J.* **46**, 96.
Gore, M. (1952). *Biochem. J.* **50**, 18.
King, E. J. (1931). *Biochem. J.* **25**, 799.
Kornberg, A. (1950). *J. biol. Chem.* **182**, 779.
Landow, H., Kabat, E. A. & Newman, W. (1942). *Arch. Neurol. Psychiat., Chicago*, **48**, 518.
Macfarlane, M. G., Patterson, L. M. & Robison, R. (1934). *Biochem. J.* **28**, 720.
Meyerhof, O. & Wilson, J. R. (1948). *Arch. Biochem.* **17**, 153.
Naidoo, D. & Pratt, O. E. (1951). *J. Neurol. Psychiat.* **14**, 287.
Naidoo, D. & Pratt, O. E. (1952). *J. Neurol. Psychiat.* **15**, 164.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Reis, J. (1937). *Enzymologia*, **2**, 110.
Richter, D. & Hullin, R. P. (1951). *Biochem. J.* **48**, 406.
Scharrer, E. & Sinden, J. (1949). *J. comp. Neurol.* **91**, 331.
Vondracek, V. (1927). *Biochem. Z.* **191**, 88.
Wolf, A., Kabat, E. A. & Newman, W. (1943). *Amer. J. Path.* **19**, 423.

Biosynthesis of Choline in the Seedling of the Chick-pea (*Cicer arietinum*)

By K. AHMAD and M. A. KARIM

Biochemistry and Nutrition Laboratory, University of Dacca, East Pakistan

(*Received* 23 *April* 1953)

The problem of methyl-group biosynthesis in the plant has not received as much attention as in the animal. Amongst important papers on the subject are those showing the formation of the methyl groups of hordenine and choline from formate by sprouting barley (Kirkwood & Marion, 1951) and of the methyl carbon of nicotine from formate and the methyl group of methionine by *Nicotiana rustica* L. (Brown & Byerrum, 1952). Barrenscheen & Pany (1941) and Barrenscheen & Valyi-Nagy (1942) observed the methylation of guanidoacetic acid to give creatine by 7-day-old wheat seedlings and also found that added methionine increased creatine synthesis sixfold to eightfold. They observed creatine synthesis to be an aerobic process where addition of hydrogen acceptors like cystine could not replace oxygen. They also noted that the transfer of the methyl group of methionine was accompanied by oxidation of the sulphur. Steensholt (1946), however, found that etiolated wheat germs were unable to use methionine for the methylation of ethanolamine to choline.

This paper describes experiments showing how the biological elaboration of the important methyl compound, choline, by the germinating seedlings of *Cicer arietinum* is influenced by certain inorganic salts, vitamins and by some organic compounds already known to be concerned with the biochemistry of methyl groups elsewhere.

EXPERIMENTAL

Technique of germination. 16–20 g. of seeds of *C. arietinum* were soaked in distilled water or in an aqueous solution of the test substance (hereafter designated as 'germinating fluid') for 12 hr. and then allowed to germinate on a sand bed in large baking dishes at room temperature (25–28°). At the end of the germination period, the seedlings, after removal of the seed coat, were dried and choline was determined.

Determination of choline. An adaptation of the methods described by Luecke & Pearson (1944) and McIntire, Sweigert and Elvehjem (1944) was used. The finely minced seedlings (1 g.) were autoclaved with 10 ml. of 3N-HCl for 2 hr. at 15 lb./sq. in. The hydrolysate after neutralization with NaOH was passed through a column of Decalso at a rate of 6 to 10 drops/min. The adsorbed choline was then eluted with two 10 ml. portions of 5% NaCl. 5 ml. of 2% ammonium reineckate in methanol were added to the eluate, which was kept at −4° for 24 hr. to allow complete precipitation of choline reineckate. The precipitate was then collected on an asbestos pad and washed three times with 2 ml. portions of ice-cold ethanol. Air was drawn through the pad until the precipitate was dry. The precipitate was then washed down with acetone and the washings were made up to 25 ml. The colour due to choline reineckate was measured in a photoelectric (Klett-Summerson) colorimeter. A standard, with 4 mg. of choline chloride, and a reagent blank were run with every determination.

Each set of experiments described below was run five times, and every determination done in triplicate. No variation over 5% was noticed and the results presented are average values.

RESULTS

During germination, the choline contents of the seedlings (Table 1, column 1) increased gradually to a maximum value of 3·78 mg. (expressed as choline chloride) per gram of the seedling (dry weight) after 96 hr. of germination and then declined. The same results were obtained in either light or darkness.

Table 1. *Distribution of choline in the cotyledon and growing part of the embryo (shoot plus radicle) of* Cicer arietinum *at various times after germination*

(The seeds were soaked in distilled water for 12 hr. and then allowed to germinate on a sand bed.)

Duration of germination (hr.)	Choline chloride content of seedling (mg./g. dry wt.)	Choline chloride (% of total) in	
		Cotyledons	Shoot plus radicle
0	1·81	100	—
24	2·35	96·2	3·8
48	2·97	82·0	18·0
72	3·51	65·5	34·5
96	3·78	49·0	51·0
120	3·58	35·0	65·0
144	2·89	25·0	75·0

Table 1 also shows the distribution of choline between the cotyledon and the growing part of the embryo (shoot and radicle) with the progress of germination. The amount in the latter increased with a concomitant decrease in the former.

Table 2 shows the effects of certain salts on the choline contents of the seedling with the progress of germination. While sodium chloride had no stimulatory effect, 0·01 M solutions of potassium chloride, potassium nitrate, ammonium chloride and ammonium nitrate stimulated choline biosynthesis considerably and after 96 hr. produced maximum increases of 10·5, 18·5, 15·9 and 27·6 %, respectively, over the control.

Table 3 shows the effects of three water-soluble vitamins, thiamine, riboflavin and ascorbic acid, added as 0·025 % (w/v) aqueous solutions. While ascorbic acid depressed choline biosynthesis by 22·9 %, riboflavin and thiamine stimulated it by 10·5 %.

Table 4 shows the effects of DL-methionine (0·1 %, w/v), creatinine (0·1 %, w/v), acetone (1 %, v/v) and methanol (1 %, v/v) which stimulated synthesis by 31·6, 29·2, 9·2 and 4·2 %, respectively, over the control.

Table 2. *Effect of certain inorganic salts on the biosynthesis of choline in the germinating seedlings of* Cicer arietinum

(The seeds were soaked for 12 hr. in 0·01 M salt solutions and then allowed to germinate on a sand bed. The figures in parentheses represent the variation from the corresponding value of the control experiment. A negative value indicates depression, a positive value stimulation, of biosynthesis.

Duration of germination (hr.)	Choline chloride content of seedling (mg./g. dry wt.) when the germinating fluid was					
	Water (control)	NaCl	KCl	KNO_3	NH_4Cl	NH_4NO_3
0	1·82	1·82	1·82	1·82	1·82	1·82
24	2·38	2·38	2·66	3·00	2·90	3·22
48	2·98	2·93	3·42	3·63	3·63	3·90
72	3·50	3·21	3·80	4·40	3·97	4·50
96	3·80	3·62 (−4·7 %)	4·20 (10·5 %)	4·50 (18·4 %)	4·40 (15·9 %)	4·85 (27·6 %)
120	3·50	3·22	3·97	4·20	4·20	4·50
144	2·88	3·22	3·63	3·81	3·97	4·10
168	2·44	3·10	3·52	3·75	3·21	3·80

Table 3. *Effect of certain water-soluble vitamins on the biosynthesis of choline in the germinating seedlings of* Cicer arietinum

(The seeds were soaked for 12 hr. in aqueous solutions of the vitamins (0·025 %, w/v) before germination on a sand bed. The meaning of the figures in parentheses is given in Table 2.)

Duration of germination (hr.)	Choline chloride content of seedling (mg./g. dry wt.) when the germinating fluid was			
	Water (control)	Thiamine	Riboflavin	L-Ascorbic acid
0	1·82	1·82	1·82	1·82
24	2·38	2·62	2·38	2·07
48	2·98	2·96	2·93	2·67
72	3·50	3·82	3·63	2·75
96	3·80	4·20 (10·5 %)	4·20 (10·5 %)	2·93 (−22·9 %)
120	3·50	4·11	3·81	2·67
144	2·88	3·43	3·42	2·68

Table 4. *Effect of certain organic compounds on the biosynthesis of choline in germinating seedlings of* Cicer arietinum

(The seeds were soaked for 12 hr. in an aqueous solution of the test substance and then allowed to germinate on a sand bed. The meaning of the figures in parentheses is given in Table 2.)

Duration of germination (hr.)	Choline chloride content of seedling (mg./g. dry wt.) when the germinating fluid was				
	Water (control)	DL-Methionine (0·1%, w/v)	Creatine (0·1%, w/v)	Acetone (1·0%, v/v)	Methanol (1·0%, v/v)
0	1·82	1·82	1·82	1·82	1·82
24	2·38	2·75	2·64	2·68	2·52
48	2·98	3·51	3·22	3·43	2·96
72	3·50	4·50	4·20	3·91	3·90
96	3·80	5·00 (31·6%)	4·91 (29·2%)	4·15 (9·2%)	3·96 (4·2%)
120	3·50	4·80	4·75	3·97	3·63
144	2·88	4·20	3·92	2·93	3·43
168	2·44	3·90	3·22	2·52	2·82

Table 5. *Effect of certain organic compounds on the biosynthesis of choline in the germinating seedlings of* Cicer arietinum

(The seeds were soaked for 12 hr. in the aqueous solutions of the test substances and then allowed to germinate on a sand bed. The meaning of the figures in parentheses is given in Table 2.)

Duration of germination (hr.)	Choline chloride content of seedling (mg./g. dry wt.) when the germinating fluid was				
	Water (control)	Nicotinamide (0·1%, w/v)	Homocystine (0·1%, w/v)	Nicotinamide (0·1%, w/v) + creatinine (0·1%, w/v)	Nicotinamide (0·1%, w/v) + acetone (1%, v/v)
0	1·81	1·81	1·81	1·81	1·81
24	2·38	2·00	2·38	2·38	2·43
48	2·99	2·38	2·68	2·68	2·64
72	3·50	2·68	2·92	3·02	3·22
96	3·80	2·69 (−29·2%)	2·98 (−21·6%)	3·22 (−15·2%)	3·80 (0%)
120	3·50	2·00	2·98	3·20	3·22
144	2·88	2·60	2·96	2·88	2·88
168	2·44	1·50	2·52	2·43	2·35

Table 5 shows the effects of nicotinamide and homocystine, and the combined effects of nicotinamide and creatinine and nicotinamide and acetone. Nicotinamide (0·1%, w/v) and homocystine (0·1%, w/v) depressed choline biosynthesis. This depression was, however, completely or partially counteracted if creatinine or acetone was applied simultaneously. Creatinine (0·1%, w/v) reduced the depressant effect of nicotinamide (0·1%, w/v) from 29·2 to 15·2%, and acetone (1%, v/v) completely abolished it.

DISCUSSION

The results (Table 1) indicating that the choline content of the seedling increased gradually during the first 96 hr. and then declined and that more of the choline accumulated in the growing part of the embryo (shoot plus radicle) with a concomitant decrease in the cotyledons as germination progressed may be regarded as indications that choline biosynthesis bears a direct relationship to the metabolic activity of the growing seedling. One may suspect that after 96 hr. one or more of the nutrients required by the growing seedling became limiting, resulting in a subsequent decline in the choline content of the seedling. The maximum amount of choline was found to have been synthesized during the first 96 hr. irrespective of the addition of potassium, ammonium, or nitrate ions (Table 2), or of methionine, creatinine, acetone or methanol (Table 4), or of thiamine or riboflavin (Table 3). It is difficult to conclude from the determination of only one product, namely choline, whether the added supply of these substances accelerated the overall process of germination in the seedling. The seeds appeared to germinate at about the same time in all cases after treatment with the germinating fluid. Potassium is a well-known activator of many enzyme systems and probably stimulated choline biosynthesis by enhancing some enzyme processes concerned in choline biosynthesis, which needed more potassium for their maximum activity. Ammonium and nitrate ions may increase the nitrogen metabolism of the seedling in favour of choline biosynthesis only by providing additional nitrogen.

The effect of riboflavin and thiamine may be traced to their role as constituents of many enzyme systems. It is not known, however, if these vitamins have similar effects on choline biosynthesis in the animal. Unlike the B vitamins, ascorbic acid is not present in the seed but is produced during germination and may change the oxidation-reduction potential of the germinating system (Williams, Eakin, Beerstecher & Shive, 1950), which may cause a depression of choline biosynthesis.

Methionine, acetone and methanol are known to give rise to choline methyl groups in the animal, although quantitative data regarding their relative potency are not available. Du Vigneaud, Chandler, Cohn & Brown (1940) demonstrated the transfer of the methyl group of methionine to choline in the rat, the reverse process, i.e. the formation of methionine from homocystine and choline having been established earlier (Du Vigneaud, Chandler, Moyer & Keppel, 1939). Sakami (1950) noted that ^{14}C-labelled acetone gives rise to the methyl carbon of choline in the rat, and it has been shown that this transformation may occur via formate (Welch & Sakami, 1950). Du Vigneaud & Verly (1950) found that methanol may serve as precursor of choline methyl groups and Du Vigneaud, Verly & Wilson (1950) demonstrated the formation of choline methyl groups from formate and formaldehyde in the rat. The contribution of formate and methionine to the methyl group of nicotine in *N. rustica* L., and of hordenine, creatine and choline in wheat seedlings has been mentioned earlier. Though glycocyamine is methylated by rat liver in the presence of methionine to give creatine (Borsook & Dubnoff, 1945), the reverse process, i.e. the formation of methionine from homocystine and creatine, could not be demonstrated (Du Vigneaud, Chandler & Moyer, 1941). The experiments presented in this paper show that DL-methionine, creatinine, acetone and methanol cause stimulation of choline biosynthesis in the seedlings of *C. arietinum* presumably by contributing to the synthesis of methyl groups. It is also shown (Table 4) that DL-methionine is better than creatinine, creatinine is better than acetone and acetone is better than methanol as a source of methyl groups.

Nicotinamide (Handler & Dunn, 1942) and homocystine (Du Vigneaud *et al.* 1940) are well-known acceptors of methyl groups in the animal. These compounds may therefore depress choline biosynthesis in the seedlings of *C. arietinum* by competing for methyl groups (Table 5). By providing additional methyl groups, creatinine and acetone reduce or eliminate this effect of nicotinamide when either of them is present together with nicotinamide during germination. The observation that acetone is more effective than creatinine in counteracting the depressant effect of nicotinamide, though the latter stimulated choline biosynthesis to a greater extent when applied alone, will need further exploration.

SUMMARY

1. The biosynthesis of choline in the germinating seedling of the chick-pea (*C. arietinum*) has been studied.

2. During the first 96 hr. the choline content of the seedling increases to a maximum value of 3·78 mg. choline chloride/g. of seedling and then gradually declines.

3. Potassium, ammonium and nitrate ions stimulate choline biosynthesis. A similar effect is produced by thiamine and riboflavin while ascorbic acid depresses choline synthesis.

4. DL-Methionine, creatinine, acetone and methanol stimulate the biosynthesis of choline by 31·6, 29·2, 9·2 and 4·2 %, respectively, after 96 hr. of germination.

5. Nicotinamide and homocystine depress choline biosynthesis in the seedling. This depression is however wholly or partially counteracted by creatinine or acetone.

REFERENCES

Barrenscheen, H. K. & Pany, J. (1941). *Biochem. Z.* **310**, 344.

Barrenscheen, H. K. & Valyi-Nagy, T. von (1942). *Hoppe-Seyl. Z.* **277**, 97–113.

Borsook, H. & Dubnoff, J. (1945). *J. biol. Chem.* **160**, 635.

Brown, S. A. & Byerrum, R. U. (1952). *J. Amer. chem. Soc.* **74**, 1523.

Du Vigneaud, V., Chandler, J. P., Cohn, M. & Brown, G. B. (1940). *J. biol. Chem.* **134**, 787.

Du Vigneaud, V., Chandler, J. P. & Moyer, A. W. (1941). *J. biol. Chem.* **139**, 917.

Du Vigneaud, V., Chandler, J. P., Moyer, A. W. & Keppel, D. M. (1939). *J. biol. Chem.* **131**, 57.

Du Vigneaud, V. & Verly, W. G. (1950). *J. Amer. chem. Soc.* **72**, 1049.

Du Vigneaud, V., Verly, W. G. & Wilson, J. E. (1950). *J. Amer. chem. Soc.* **72**, 2819.

Handler, P. & Dunn, W. J. (1942). *J. biol. Chem.* **146**, 357.

Kirkwood, S. & Marion, L. (1951). *Canad. J. Chem.* **29**, 30.

Luecke, R. W. & Pearson, P. B. (1944). *J. biol. Chem.* **155**, 508.

McIntire, J. M., Sweigert, B. S. & Elvehjem, C. A. (1944). *J. Nutr.* **28**, 219.

Sakami, W. (1950). *Fed. Proc.* **9**, 222.

Steensholt, G. (1946). *Acta Physiol. scand.* **11**, 136–40.

Welch, A. D. & Sakami, W. (1950). *Fed. Proc.* **9**, 245.

Williams, R. J., Eakin, R. E., Beerstecher, E. jun. & Shive, W. (1950). *The Biochemistry of B vitamins*, Amer. chem. Soc. Monograph no. 110, p. 21. London: Chapman; New York: Reinhold.

Quantitative Estimation of Ascorbic Acid and Related Substances in Biological Extracts by Separation on a Paper Chromatogram

By YU-TUAN CHEN, F. A. ISHERWOOD and L. W. MAPSON
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research

(*Received* 15 *April* 1953)

In studies on the synthesis of L-ascorbic acid in plants and animals the need arose for a method permitting separation and quantitative estimation of closely related enediol compounds such as D-araboascorbic and L-ascorbic acids. Simple titration methods using 2:6-dichlorophenolindophenol (Harris & Ray, 1935; Birch, Harris & Ray, 1933; Harris & Olliver, 1942) will not differentiate between closely related enediols, and even more specific methods, based on the different rates of condensation of enediols with formaldehyde (Lugg, 1942; Mapson, 1943; Snow & Zilva, 1943), will not distinguish between those with the same ring structure. It seems clear that a preliminary separation must precede the estimation of the individual enediols. Such a separation has been described by Mapson & Partridge (1949). These authors have shown that L-ascorbic acid, D-araboascorbic acid, the enol form of 3-hydroxytetrahydrofuran-2:4-dione (hydroxytetronic acid), reductic acid, and reductone can be separated on a paper chromatogram using *n*-butanol:acetic acid:water, or phenol:acetic acid:water, as solvents. Their experiments were confined to the qualitative separation of made-up mixtures of pure compounds, and no attempts were made to estimate the enediols after separation on the paper chromatograms. The problem of separating L-ascorbic acid from D-araboascorbic acid in extracts from biological material was not touched and it was suggested that the separation might be difficult. This opinion was based on the known instability of the enediols and their low concentration in extracts from biological materials.

The method now described uses paper chromatograms not only to effect the final separation of the enediols but also to achieve a preliminary concentration from the bulk of the other compounds present in the extract. Since enediols are known to be most stable in strongly acid solution and when heavy metals are absent, both the initial extract of the biological material and the two chromatographic solvents used were saturated with oxalic acid. Oxalic acid was used because it readily forms complexes with most heavy metals and because it forms a component of two very stable chromatographic solvents (*n*-butanol:water:oxalic acid, and phenol:water:oxalic acid) which will separate most of the common enediol compounds. The presence of oxalic acid, however, does not prevent a slow decomposition of the enediols, which is particularly noticeable in weak solutions. This occurs even when the solutions are stored in the dark, but is more pronounced when they are exposed to light. The same is true of solutions in metaphosphoric acid, so that it appears to be independent of the compound used to inactivate the heavy metals. This slow breakdown inevitably makes it difficult to devise a really quantitative method. However, the present method, whilst not having a very high accuracy, does give an approximately quantitative estimate of the enediols in extracts of biological material. It can usefully be employed to confirm the reliability of the simple titration methods which are commonly used for the estimation of L-ascorbic acid in extracts of biological materials.

EXPERIMENTAL

Materials. The L-ascorbic and D-araboascorbic acids were purchased from Roche Products Ltd., Welwyn. Titration with 2:6-dichlorophenolindophenol according to the method of Harris & Olliver (1942) gave results within 1% of the theoretical. The hydroxytetronic and reductic acids were a gift from Prof. Reichstein, Basel, and the reductone a gift from Prof. Bergel, Roche Products Ltd. The oxalic acid was an A.R. sample.

The two solvents used for development of the paper chromatograms were as follows. The first was prepared by shaking *n*-butanol (60 ml.) with water (40 ml.) and excess of oxalic acid in a separating funnel and allowing to stand at 20°. The water-poor phase (upper layer) was used as solvent. The second was prepared by dissolving water (10 g.) in phenol (50 g.), filtering the solution through charcoal to remove coloured quinone compounds present, and then adding an excess of oxalic acid. The mixture was stored at 20° and the water-poor phase (lower layer) used as solvent.

A stock solution of 2:6-dichlorophenolindophenol in water was prepared by dissolving 0·8 g. of the dyestuff in 1 l. of water. To this solution was added a slight excess of potassium oxalate. It was allowed to stand overnight and then centrifuged to remove calcium oxalate. For the estimation of ascorbic acid the stock solution was diluted to a concentration of 0·04 g./l. by adding 0·25 M-Na_2HPO_4 in glass-distilled water.

Chromatography. L-Ascorbic acid and related compounds were separated on paper chromatograms before being quantitatively determined. The apparatus and general conditions were similar to those described for the separation of the sugars by Isherwood & Jermyn (1951). *n*-Butanol: water: oxalic acid was used for the general separation of the enediols from accompanying substances in plant or animal extracts and phenol: water: oxalic acid for the final separation of L-ascorbic acid from D-araboascorbic acid. These two solvents are referred to below as the butanol and phenol solvents, respectively.

The filter paper used was Whatman no. 1 for qualitative work and no. 3 for preparative work. In all cases the papers were dipped into 2% (w/v) aqueous oxalic acid and then dried in air.

In qualitative examinations the positions of the enediols on the paper chromatograms were located by spraying with 0·08% (w/v) aqueous 2:6-dichlorophenolindophenol. The enediols appear as white spots against a pink background. The limit of detection is 1–2 μg./cm.^{2} on Whatman no. 1 paper. In quantitative analyses a 'guide' chromatogram was run alongside the main chromatogram and only this guide chromatogram was sprayed. The enediols on the main chromatogram were located by comparison.

Analytical procedure. This is described for the quantitative estimation of L-ascorbic acid in urine and in cress seedlings. A qualitative examination is similar except that less care need be taken to ensure a complete recovery of L-ascorbic acid during the preliminary isolation from the plant or animal source. The description is given in terms of L-ascorbic acid but applies equally to D-araboascorbic acid and related compounds.

With urine the procedure varied somewhat, depending on the amount of L-ascorbic acid present. For samples containing less than 1 mg./ml. it was necessary to isolate the L-ascorbic acid from the bulk of the other substances present before attempting to estimate it by the standard method.

The rats were housed in standard metabolism cages, and the urine collected in a bottle containing sufficient solid oxalic acid to ensure that it was saturated with oxalic acid throughout the period of collection. All the subsequent operations were carried out in the dark or in a dim light. Even exposure to diffused daylight was avoided as much as possible. This urine (0·4 ml.) was applied to the starting line of an oxalic acid-treated Whatman no. 3 paper as a streak about 20 cm. long. The paper was then irrigated with the butanol solvent until the solvent front had moved about 30 cm. (16 hr.). After development it was removed from the jar and dried in a current of air at room temperature. A transverse strip of paper (30 × 3 cm.), corresponding to the L-ascorbic acid on the guide chromatogram, was cut out of the main piece of paper, and extracted chromatographically with glass-distilled water until about 2 ml. of extract had been obtained. In cases where the concentration of L-ascorbic acid in the urine was very low, a number of chromatograms were run and the extracts of L-ascorbic acid from each combined until about 100 μg. had been obtained. Since the urine from normal rats contained about 50 μg./ml., six chromatograms carrying 2·5 ml. of the urine gave sufficient for the present purpose. The combined extracts were then saturated with H_2S and treated with 10% (w/v) aqueous calcium acetate until most of the oxalic acid had been removed. Adding calcium acetate until the pH was about 2·0 left just sufficient oxalic acid to stabilize the L-ascorbic acid through subsequent operations. The solution was then centrifuged to remove calcium oxalate. The supernatant was evaporated to dryness *in vacuo* over P_2O_5. Leaving the solution in a small beaker overnight in a vacuum desiccator was sufficient. The residue was dissolved in 0·1 ml. of glass-distilled water. 10 μl. of this solution from a micropipette were applied to the starting line of an oxalic acid-treated Whatman no. 1 paper and the paper was developed with the phenol solvent for 20 hr. (the solvent front has moved about 35 cm.). The appropriate piece of paper was cut out and the L-ascorbic acid estimated.

For samples that contain amounts of L-ascorbic acid larger than 1 mg./ml., the urine was applied directly to the starting line of an oxalic acid-treated Whatman no. 1 paper and developed with the phenol solvent as described above.

For cress seedlings the procedure was similar to that devised for urine except that the extract to be applied to the starting line was obtained from 100 cress seedlings (wet wt. 2 g. after 120 hr. germination in the dark) which were ground with 0·2 g. of crystalline oxalic acid in a mortar at 0° and then centrifuged.

Colorimetric estimation. The paper (3 × 3 cm.) containing the L-ascorbic acid (about 15 μg.) was folded twice in one direction and once in the other, using glass rods. Metal forceps were avoided because contamination with heavy metals would be likely to cause oxidation of the L-ascorbic acid. The folded paper was inserted into a clean, dry colorimeter tube and 3 ml. of a 3% (w/v) aqueous solution of oxalic acid added. An EEL single-photocell colorimeter was used (Evans Electroselenium Ltd., Harlow, Essex). This was very convenient because the whole estimation could be carried out in one of the standard colorimeter test tubes (8 × 1·5 cm.). The solution was agitated by passing a slow stream of N_2 for 2 min. The paper was then removed with a glass rod. To the solution was added 4 ml. of the freshly prepared 0·004% solution of 2:6-dichlorophenolindophenol. The solution in the colorimeter tube was mixed by rocking gently and then the colour measured within 30 sec. of adding the dye. A green-blue filter was used (Ilford 623 with maximum transmission at 490 mμ). 15 μg. of L-ascorbic acid gave a reading of about twelve divisions (blank 62, actual reading 50). The change in colour was proportional to the amount of L-ascorbic acid present. The dye was standardized against a freshly prepared solution of L-ascorbic acid.

RESULTS

Table 1 shows the R_F values obtained with the two solvent mixtures for different enediol compounds.

The phenol solvent gives a clear-cut separation of L-ascorbic acid from D-araboascorbic acid.

Table 1. *Separation of compounds related to L-ascorbic acid on paper chromatograms*

Substance	R_F value on Whatman no. 1 paper at 20°: *n*-Butanol sat. with water and oxalic acid	Phenol sat. with water and oxalic acid
L-Ascorbic acid	0·29	0·43
D-Araboascorbic acid	0·30	0·50
Hydroxytetronic acid	0·61	0·68
Reductic acid	0·60	0·81
Reductone	0·59 (trailing)	0·68

Table 2. *The effect of light on the recovery of* L-*ascorbic acid from paper chromatograms developed with the phenol solvent* (*see text*)

Amount added (μg.)	Amount found (μg.)	Recovery (%)
	In daylight	
12·0	10·1	83
24·5	20·6	84
36·5	30·3	83
	In the dark	
12·0	10·8	90
20·0	18·4	92
32·5	30·2	93
40·5	39·0	94

The effect of light on the destruction of ascorbic acid added to paper chromatograms, and then extracted and estimated as described previously, is illustrated in Table 2. Parallel experiments were carried out in which, during the normal handling necessary, the papers were exposed close to the light from a north window during March–April, and also in the absence of light. The papers were not exposed to light during the development of the chromatogram, nor during any extraction procedures. The results indicate that exposure to diffuse daylight caused an extra loss of about 10 %.

The complete procedure for the quantitative estimation of the enediols in extracts of biological materials was tested by adding known amounts of L-ascorbic acid and D-araboascorbic acid to rat's urine, and to an extract of cress seedlings. In some experiments, where the amount of enediol was greater than 1 mg./ml., the extract was examined, without any preliminary concentration, on a paper chromatogram using the phenol solvent. The enediols were then estimated from this chromatogram. The results obtained are given in Table 3.

The results given in Table 3 indicate that the loss of enediol varies between 10 and 20 % depending on the concentration in the original extract. A similar loss occurs when pure solutions of the enediols are kept in the dark for the same period (total time 3 days) so that the observed recovery is regarded as satisfactory. In practice, an estimate of the loss was obtained by carrying out a parallel determination on a pure solution containing similar amounts of the enediol and then a correction applied to the figures observed for the extract from the biological material. The figures in Table 3 were not corrected in this manner but are given to illustrate errors inherent in the method.

SUMMARY

1. L-Ascorbic acid and related compounds have been separated on a paper chromatogram using two new solvent mixtures, *n*-butanol saturated with water and oxalic acid, and phenol saturated with water and oxalic acid. The phenolic solvent will give a clear-cut separation of L-ascorbic acid from D-araboascorbic acid.

2. A quantitative procedure is described for the estimation of enediols from plant and animal tissues. Enzymes are inactivated by saturating the extract with oxalic acid and the enediols are stabilized throughout subsequent operations by maintaining a high concentration of oxalic acid. The enediols are estimated after separation on a paper chromatogram by a colorimetric method using a solution of 2:6-dichlorophenolindophenol in 0·25 M-Na_2HPO_4. Recovery of L-ascorbic acid and D-araboascorbic acid added to rat's urine, and extracts of cress seedlings was 85 % even at the lowest concentration examined (0·5 mg./ml. of urine or cress-seedling extract).

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

Table 3. *The recovery of* L-*ascorbic acid and* D-*araboascorbic acid added to rat urine and to an extract of cress seedlings* (*uncorrected*)

Biological material	Procedure	L-Ascorbic acid present initially (mg./ml.)	Amount added		Amount found	
			L-Ascorbic (mg./ml.)	D-Arabo-ascorbic (mg./ml.)	L-Ascorbic (mg./ml.)	D-Arabo-ascorbic (mg./ml.)
Rat's urine	Full	0	0·6	0·6	0·51	0·52
Rat's urine	Preliminary concentration omitted	0	2·00	2·10	1·86	1·95
Cress seedlings	Full	0·30	0·53	0·74	0·72	0·65
Cress seedlings	Preliminary concentration omitted	0·39	2·23	2·50	2·49	2·37

REFERENCES

Birch, T. W., Harris, L. J. & Ray, S. W. (1933). *Biochem. J.* **27**, 90.
Harris, L. J. & Olliver, M. (1942). *Biochem. J.* **36**, 155.
Harris, L. J. & Ray, S. W. (1935). *Lancet*, **1**, 71.
Isherwood, F. A. & Jermyn, M. A. (1951). *Biochem. J.* **48**, 515.
Lugg, J. W. H. (1942). *Aust. J. exp. Biol. med. Sci.* **20**, 273.
Mapson, L. W. (1943). *J. Soc. chem. Ind., Lond.*, **62**, 223.
Mapson, L. W. & Partridge, S. M. (1949). *Nature, Lond.*, **164**, 479.
Snow, G. A. & Zilva, S. S. (1943). *Biochem. J.* **37**, 630.

Separation and Estimation of Organic Acids on Paper Chromatograms

By F. A. ISHERWOOD
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research

AND C. S. HANES*
Agricultural Research Council Unit of Plant Biochemistry, Botany School and Biochemistry School, University of Cambridge

(*Received* 15 *April* 1953)

In the present paper a method is described for the qualitative separation and quantitative estimation of the ammonium salts of a wide variety of acids on paper chromatograms using single-phase mixtures of *n*-propanol and concentrated aqueous ammonia as the developing solvents. The method for the qualitative separation of organic acids was briefly mentioned in a previous paper describing the separation of phosphoric esters on paper chromatograms (Hanes & Isherwood, 1949), and since then a number of workers (Brown & Hall, 1950; Brown, 1950; Hiscox & Berridge, 1950; Kennedy & Barker, 1951; Brown, 1951; Long, Quayle & Stedmann, 1951) have employed basic solvent mixtures to effect a qualitative separation of organic acids.

The method developed for the quantitative determination of the ammonium salts of organic acids is based upon a simple colorimetric procedure, and is accurate to $\pm 10\%$ when about 50 μg. of organic acid is being determined. The method rests upon the fact that under suitable conditions the colour of a selected indicator is unaffected by the presence of ammonia, so that the ammonium salts of organic (and other) acids behave as free acids, the colour of the indicator being diminished in proportion to their concentrations. The principle has been applied to determining both volatile and non-volatile organic acids on paper chromatograms.

The same principle could obviously be applied to the determination of organic bases which could be separated as salts of a weak acid (e.g. by separating chromatographically in an appropriate solvent containing acetic acid). The acetates of the bases could then be determined by their effect upon the colour of an indicator (of pK 2–3) under conditions in which it would be unaffected by the presence of acetic acid in small amount.

For most of the experiments described in the present paper artificial mixtures of organic acids were used, but satisfactory results were obtained on extracts of biological materials by a modified procedure in which the acids were first separated from the bulk of the other solutes by a method using a silica gel column (Isherwood, 1946) before being examined on the paper chromatogram.

Since this work was completed, Reid & Lederer (1952) have published a method for the estimation of the lower fatty acids (C_2–C_7) based on the area of the spot on a chromatogram.

EXPERIMENTAL

Reagents

The various organic acids used in this study were examined on a paper chromatogram using *n*-propanol: concentrated aqueous NH_3 as solvent and only those which appeared to be homogeneous were accepted.

For the quantitative analysis of the acids on paper the propanol used was purified as follows. It was refluxed for 1 hr. with solid NaOH, and then distilled through a fractionating column, rejecting the first fraction (b.p. range, 93–95°) and collecting the next fraction (b.p. range, 96–98°). Commercial propanol, which is adequate for the qualitative separation of the acids, appears to contain traces of an organic acid which seriously affects the blank value of the paper in the method described below. The concentrated NH_3 was A.R. grade.

The ethanol used for preparing the thymol blue-glycine buffer reagent was purified by refluxing for 1–2 hr. over solid NaOH and distilling in an all-glass apparatus. Ordinary absolute ethanol contains sufficient aldehyde and acidic impurities to affect the blank values of the thymol blue reagent.

Analytical procedures

Chromatography. The apparatus and conditions used for the development of the paper chromatograms were essentially the same as those described by Hanes & Isherwood (1949) for phosphoric esters. Solutions of the organic acids, normally as ammonium salts and in concentrations not exceeding 0·3 N, were spotted at 3 cm. intervals along the starting line of a sheet of filter paper by means of a micropipette of volume about 4 μl. This consisted of a thin-walled spindle-shaped capillary tube conveniently mounted at the mouth of a glass tube. It is essential to calibrate the pipette for the particular solution to be used, if the surface tension (due to the presence of organic solvent) or viscosity are appreciably different from those for water.

The paper chromatograms were developed with a single-phase mixture of *n*-propanol and aqueous NH_3. The actual proportions of the components varied with the group of

* Present address: Department of Biochemistry, Toronto, Canada.

acids to be separated; solvents with more water were useful in separating the acids with more than one hydroxyl or carboxyl group. A very convenient mixture for the lower fatty acids was *n*-propanol: conc. aqueous NH_3 (sp.gr. 0·880) (70:30, v/v). For the dicarboxylic acids such as tartaric and malic, the solvent contained *n*-propanol: water: conc. NH_3 (60:20:20, v/v).

Spraying reagents. To detect the various organic acids the developed chromatograms were sprayed (3–5 ml./100 cm.²) with one of the following solutions:

(1) *Thymol blue.* 0·1% (w/v) in water to which sufficient 0·1N-NaOH had been added to render the solution deep blue, pH 10. When this was sprayed on to a paper in a CO_2-free atmosphere, the paper was coloured light blue. The ammonium salts of the acids showed up as yellow spots on a deep-blue background, cations other than ammonium showing up as spots with a deeper blue colour than the background. For spraying, the papers were suspended in an accumulator jar by clamping one end of the paper between two glass plates which closed the open end. The jar had a hole bored in a central position through one of the vertical sides so that the orifice of a spray could be inserted and the paper sprayed. The developed chromatograms were suspended in the jar for at least 10–20 min. while a stream of CO_2-free air was blown through before being sprayed. The compressed air operating the spray was also free from CO_2. Once the papers were removed from the jar the background colour faded but lasted long enough for the positions of the acids to be outlined. The indicator is very sensitive and will even detect phenols on the paper.

(2) *Ammoniacal silver nitrate.* This was prepared by diluting 0·1M-$AgNO_3$ with one-fifth its vol. of conc. aqueous NH_3. After spraying, the papers were heated at 105° for periods up to 30 min. The reagent is very sensitive and reacts with a wide variety of substances. Reducing substances such as sugars, aldehydes, hydroxy acids and phenols give dark brown-violet spots, the shade of which is often characteristic of the type of substance. Some non-reducing acids inhibit the colour developed by the paper itself and the position of these acids is revealed by a white spot on a light-brown background. Salts and amino acids also affect the reagent, but usually to a much smaller extent. This reagent is not specific for acids. Chromatograms sprayed with this reagent are usually compared with similar chromatograms sprayed with the thymol blue reagent in order definitely to identify unknown compounds as acids. The papers sprayed with the silver reagent are useful as permanent records.

Purification of filter paper. The filter paper used was purified by chromatographic washing with 2N acetic acid, distilled water and 10N-NH_3. Purification of the paper did not affect the general movement of the various acids on the chromatogram but did give distinctly better resolution. The spots showed less tendency to trail, 'ghost' spots were not left behind on the starting line and the sensitivity of methods for the location of acid spots was improved because the paper had been freed of soluble acidic and reducing impurities. For quantitative work the use of washed paper was essential. Whatman no. 1 paper was used for both qualitative and quantitative work, and no. 3 for preparative work.

The apparatus used for the chromatographic washing of filter papers in the present study consisted of a divided Perspex trough which would take a block of about 60 sheets of Whatman no. 1 paper (11·25 in. wide). The design of the trough was such that when the two sides were firmly clamped together the block of papers was held firmly without any gaps or spaces through which liquid could percolate. The arrangement is shown in Fig. 1. For assembly the block of paper was bevelled very slightly with a sharp razor at A and A_1 and then inserted into the slot in the trough. The sides were then clamped together, locating bolts at each end ensuring that the two halves were correctly positioned. The slight gaps which might be present at A and A_1 were closed by ramming a little filter-paper pulp into the crevices. The trough with the block of paper was then suspended in a conveniently sized tank and the trough filled with the washing liquid. The whole apparatus was covered with Alkathene sheeting to keep out dust. The fit of the block of paper in the trough was tested by measuring the flow of liquid through the paper; about 1 l. should percolate every 24 hr. The papers were washed until no more soluble material was removed. This was tested by chromatographically extracting a small piece of the paper (5 × 20 cm.) with 10 ml. of 10N-NH_3 and evaporating the extract to dryness. Less than 0·3 mg. of residue should remain.

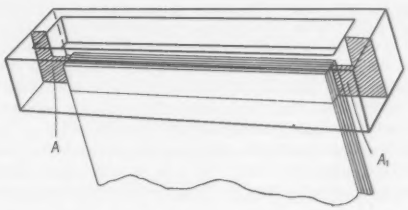


Fig. 1. Apparatus for chromatographic washing of filter paper. For explanation see text.

Quantitative estimation of the ammonium salts of organic acids. The indicator solution was prepared by adding 1 ml. of a 0·05M solution of the sodium salt of glycine (made by dissolving 3·75 g. glycine in 1 l. of 0·05N-NaOH, free from carbonate) to 450 ml. of 35% (v/v) aqueous ethanol containing 8 mg. of thymol blue. The solution was stirred by a stream of CO_2-free air. To the solution was added 1·4 ml. of 0·1N-NaOH, which should bring the intensity of the blue colour to about 80% of the maximum value (i.e. that observed when excess of NaOH is added) as measured under standard conditions in the colorimeter. The solution was stored in the 500 ml. reservoir attached to a 10 ml. burette with an automatic zero point. The bottle and burette were protected against the entry of CO_2.

The procedure in the case of a synthetic mixture or a relatively pure solution of the ammonium salts of the acids was as follows: 4 μl. or an appropriate quantity to give a reading in the colorimeter equivalent to about 40% neutralization of the indicator-buffer solution, was applied to the starting line of a paper chromatogram (Whatman no. 1, washed). The paper was developed with propanol: aqueous NH_3; the proportions depended on the acids to be separated. After development, the paper chromatograms were suspended in accumulator jars and a stream of dry CO_2-free air was blown through for at least 2 hr. The dry papers were then cut into strips corresponding to the chromatograms of the various spots originally applied to the starting line and the individual acids on these located by reference to a guide chromatogram. The piece of paper (usual size 3 × 3 cm.) containing the ammonium salt of the acid was cut out of the chromatogram and then folded twice in one direction and

once in the other, using a clean forceps. Each folded paper was inserted into a clean dry colorimeter tube and 3 ml. of 35% (v/v) aqueous ethanol were added. A slow stream of CO_2-free nitrogen or air was bubbled through the liquid by means of a fine capillary tube inserted into the colorimeter tube. This stream of CO_2-free gas was continued throughout subsequent operations until just before the colour was measured in the colorimeter. In addition, the tube was usually partly closed by a clean rubber bung to prevent the entry of CO_2. After 5 min. the paper was carefully lifted out of the liquid with a clean platinum wire, pressed against the side of the tube with a clean glass rod and then removed. 4 ml. of the thymol blue reagent were added, the tip of the burette being inserted well into the colorimeter tube. After 1 min. the capillary tube was withdrawn and the test tube promptly closed with the bung. The contents were shaken and the colour was measured in the colorimeter, using a green filter (Ilford 626).

The measurement of the blank must be carried out on a piece of paper from the same chromatogram. Even washed paper did not behave the same before using as a chromatogram as afterwards. In a typical example the blank readings were as follows: without any paper, 65; with the addition of paper from a freshly dried (in absence of CO_2) paper chromatogram, 68; with addition of washed paper stored in the laboratory, 63. The discrepancy between the papers is serious in the case of small amounts of acid.

In the case of extracts of biological material, the acids were first separated from the bulk of the other solutes present before estimating by the procedure described above. The procedure using silica-gel columns described by Isherwood (1946) was employed to extract all the acids, volatile and non-volatile. The plant juice acidified with H_2SO_4 was absorbed into silica gel and extracted in the form of a column with 50% (v/v) *n*-butanol: $CHCl_3$. The solvent which issued from the column was then neutralized with a slight excess of 20N-NH_3. The aqueous phase which separated contained a concentrated solution of the ammonium salts of all the acids and was used without further purification for the procedure described above.

Chromatographic extraction of filter-paper strips. The apparatus used was similar in principle to that ordinarily employed for paper chromatography except that the trough was made of thin Perspex sheet and could be readily removed from the chromatogram jar. The paper strips were suspended from the trough and the lower end of each paper cut to a point. A small stainless steel or glass clip was attached to the point. The liquid percolating through the paper dripped into a cup supported by an adjustable mounting.

RESULTS

Behaviour of carboxylic acids on paper chromatograms

A large number of mono-, di-, and tri-carboxylic acids were examined using different mixtures of *n*-propanol and concentrated aqueous ammonia as solvents. The R_F values are presented in Table 1.

Table 1. *R_F values of organic acids on paper chromatograms using mixtures of* n-*propanol and concentrated ammonia as developing solvents*

(Temp. 20°, Whatman paper, no. 1, washed. The mixtures of *n*-propanol: conc. aqueous ammonia (sp.gr. 0·880) used as solvents were (in vol.): no. 1, 90:10; no. 2, 80:20; no. 3, 70:30; no. 4, 60:40.)

		R_F value in solvent no.			
Monocarboxylic acids	x	1	2	3	4
$H.(CH_2)_x.COOH$	0	—	—	0·37	0·52
	1	0·13	—	0·37	0·52
	2	0·25	—	0·48	0·61
	3	0·33	—	0·57	0·69
	5	0·44	—	0·69	0·80
	7	0·52	—	0·73	0·84
	9	0·58	—	0·78	0·86
	13	0·62	—	0·82	0·88
Dicarboxylic acids					
$HOOC.(CH_2)_x.COOH$	1	—	0·04	0·09	0·23
	2	—	—	0·13	0·30
	3	—	0·07	0·16	0·34
	4	—	0·083	0·19	0·39
	6	0·02	0·18	0·29	0·49
	7	0·04	0·24	0·33	0·58
	8	0·07	0·28	0·44	0·65
Other acids					
Glycollic		—	—	0·26	0·39
Lactic (DL-)		—	—	0·395	0·48
Glyceric (DL-)		—	—	0·21	0·38
Maleic (*cis*)		—	—	0·08	0·21
Fumaric (*trans*)		—	—	0·11	0·23
Malic (DL-)		—	—	0·06	0·195
Tartaric (L-)		—	—	0·03	0·15
Tricarballylic		—	—	0·01	0·12
Aconitic (*trans*)		—	—	0·00	0·10
Citric		—	—	0·00	0·07

The results show that the amount of water in the solvent influences the R_F values in a regular manner; increasing amounts increase the R_F but the order in which the acids separate is unchanged. This is in agreement with the observations of Isherwood & Jermyn (1951) on the sugars. These authors showed that the plot of $\log_{10}\left(\frac{1}{R_F}-1\right)$ against $-\log_{10}(N)$, where N was the molar concentration of water in the solvent, was a straight line for each sugar. It is probable that a similar relationship exists for the ammonium salts of the organic acids but the data obtained in the present study were insufficient for a more detailed analysis.

Martin (1949) suggested the use of $RT \ln \alpha$ (where α is the partition coefficient) for comparing the chromatographic behaviour of members of a series of compounds, since the function might be expected to be the sum of contributions from all the constituent groups of a molecule, assuming that the effect of each of these on the distribution isotherm is independent of the others. Accordingly, in Fig. 2 values of $\log_{10}\left(\frac{1}{R_F}-1\right)$, which is proportional to the above function, have been plotted against the number of carbon atoms for the members of the two homologous series of organic acids for which data are available.

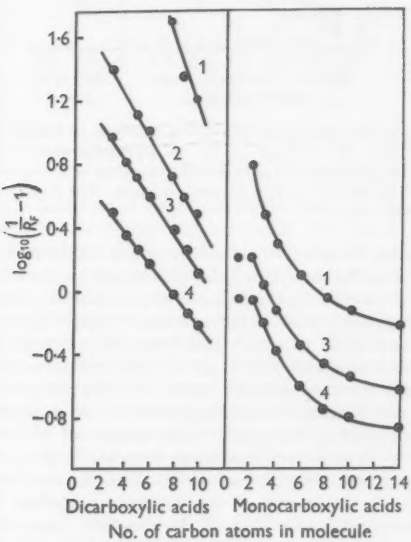


Fig. 2. Relationship between $\log_{10}\left(\frac{1}{R_F}-1\right)$ and the number of carbon atoms in the molecule for the monocarboxylic acids $CH_3.(CH_2)_n.COOH$, and for the dicarboxylic acids $HOOC.(CH_2)_n.COOH$. The mixtures of *n*-propanol : conc. NH_3 used as solvent were (by vol.): (1) 90:10; (2) 80:20; (3) 70:30; (4) 60:40.

It will be seen that the results for the dicarboxylic acids adhere closely to the relationship expected from the theory of Martin, the points for each solvent mixture falling closely upon a straight line. The results for the monocarboxylic acids, on the other hand, show a marked deviation from the expected linear relationship. For this series the value $\log_{10}\left(\frac{1}{R_F}-1\right)$ tends to approach a limiting value as the number of carbon atoms increases, an effect observed with all three solvents investigated. Other investigations showed that the form of this relationship was unaffected by the type of filter paper used (Whatman nos. 54, 541 and 1 unwashed were tried) and by the presence or absence of carbon dioxide in the chromatogram jar: there is no doubt that it is a characteristic of this homologous series of acids, the detailed interpretation of which is likely to prove of some theoretical interest. It should be noted that the decrease in hydrophilic character with increased number of methylene groups falls off to very small values in the region C_6–C_8; it may be significant that the ammonium salts of the acids begin to show the characteristic properties of soaps when this molecular size is reached. The unexpected chromatographic behaviour may thus reflect a marked change in the association of the molecules of the ammonium salts in the system (cf. Duin, 1953).

Quantitative analysis of ammonium salts of organic acids on paper chromatograms

The basis of the method is that if an acid is added to a neutralized solution of an acid indicator of which the anion is distinctively coloured, then the colour due to the anion is inversely proportional to the amount of acid added between 20 and 80% neutralization. It is assumed that the acid is very much stronger than the indicator acid and that the pH range of the indicator in water does not lie above 10. Ammonium salts behave in the same way if a suitable indicator is used with a pH range above 10·0. This pH was determined by the highest concentration of free ammonia which might be expected (1×10^{-4} M) as a result of the reaction of the ammonium 10*n* on a paper chromatogram with the neutralized indicator. However, in practice the concentration of alkali needed to neutralize such indicators (1×10^{-3} M) made them too insensitive for the present purpose. A more convenient method was to use an indicator such as thymol blue (pK 9·6 in 35% (v/v) aqueous ethanol) in conjunction with an aqueous-ethanolic medium. The effect of the ethanol was to depress the dissociation constant of the ammonium hydroxide liberated and at the same time raise the apparent dissociation constant of the indicator. The

combined effect was to decrease the effect of the liberated ammonia on the indicator to such an extent that it did not interfere seriously with estimation of the ammonium salts. Thymol blue was chosen as indicator because of its distinctive colour change from blue to yellow. In practice the indicator was combined with a colourless buffer (sodium glycinate) which had similar characteristics, the amount of indicator being set by the colorimeter used and the buffer by the strength of the ammonium salts to be measured. Glycine was chosen because its pK_A (9·8 in 35 % (v/v) aqueous ethanol) was very close to that of thymol blue (9·6 in the same solvent) and because it did not interact with any of the acids or with sugars. Boric acid, which was used in some of the early experiments, is known to give strongly acid complexes with hydroxy compounds which may be present in crude extracts from plant materials. The standard thymol blue-glycine reagent was designed for use with an EEL single photocell colorimeter (Evans Electroselenium Ltd., Harlow, Essex) and for the estimation of quantities of acid from 20 to 120 μg. The results of adding sulphuric acid, ammonium sulphate and sodium hydroxide to the standard reagent are shown in Fig. 3. It is clear that the addition of ammonium sulphate has not the same effect as the addition of sulphuric acid but that the difference down to 20 % neutralization is small. Below 15 % neutralization the liberated ammonia influences the results. Between 20 and 80 % neutralization the curve is straight and this constitutes the working range of the method. Above 80 % the curve bends sharply because at the very alkaline pH a considerable excess of sodium hydroxide was needed to complete the neutralization. The small reading in acid solution was due to the yellow colour of the acid form of the indicator. The effect of adding ammonium sulphate in the presence of an excess of free ammonia is shown in Fig. 3 for reagents made up in 35 and 45 % (v/v) aqueous ethanol. The amount of ammonia added was equivalent to the maximum amount of ammonium sulphate which could be measured by the reagent (equivalent to 2·44 μl. of 0·5N). It is clear that excess of ammonia noticeably affects both the reagent made up in 35 % aqueous ethanol and that made up in 45 % (v/v) aqueous ethanol but that the effect on the latter is much less marked. Free ammonia is not present in any quantity if the paper chromatograms are dried thoroughly and then the reagent made up in 35 % (v/v) aqueous ethanol is satisfactory. In addition, the linear portion of the curve is very much longer so that a wider range of concentrations can be measured. The quantitative results described later in this paper were all measured using this reagent.

The general effect on the subsequent paper chromatograms of washing the filter paper used has

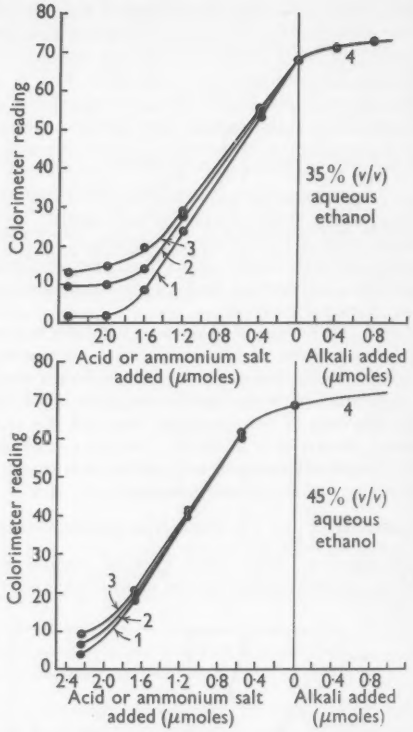


Fig. 3. The effect on colorimeter readings of adding (1) H_2SO_4, (2) $(NH_4)_2SO_4$, (3) an equimolar mixture of $(NH_4)_2SO_4$ and NH_3, (4) NaOH to the standard reagent in 35 % and 45 % (v/v) aqueous ethanol. For details see text.

already been briefly described in the Experimental section. In the case of the quantitative estimation of the acids, washed paper was essential. Early experiments with Whatman no. 1 which had not been washed or which had been washed with 2N acetic acid and water gave very erratic results. Acids such as glyceric acid were impossible to estimate because there appeared to be a soluble compound in the paper which interfered with the reaction with the thymol blue reagent. Washing the paper with *n*-propanol:conc. aqueous ammonia (70:30, v/v) removed this inhibitor and then the ammonium salts could be readily estimated. Washing with a mixture of equal volumes of concentrated ammonia and water was equally effective and considerably cheaper.

The results for representative acids in different concentrations on washed Whatman no. 1 paper using purified *n*-propanol:conc. aqueous ammonia as solvent (70:30, v/v) are shown in Table 2.

Table 2. *Estimation of the ammonium salts of various acids on paper chromatograms (washed Whatman no. 1) using purified n-propanol:conc. aqueous ammonia (sp.gr. 0·880) as solvent (70:30, v/v) (single estimations)*

Acid	Amount added (μg.)	Amount found (μg.)	
Malic	16·6	16·6	
	33·2	34·8	
	66·4	70·0	
	83·0	81·0	
Succinic	14·6	13·8	—
	29·5	26·2	27·8
	44·2	44·0	48·0
	59·0	60·6	56·0
Acetic	24·2	22·4	
	48·4	45·6	
	72·6	71·5	

Table 3. *Analysis of mixtures of acetic, succinic and citric acids on washed Whatman no. 1 paper using n-propanol:conc. aqueous ammonia (sp.gr. 0·880) (70:30, v/v) as solvent (single estimations)*

Acetic	Succinic	Citric
Amount of acid added* (μg.)		
24·2	47·6	38·6
48·4	71·0	72·5
72·6	71·0	38·6
Amount of acid found (μg.)		
26·3	46·4	37·4
45·0	69·5	77·0
71·5	65·3	36·1

* The weights refer to free acids, although they were added as ammonium salts.

The amount of acid calculated from the colorimeter reading was not corrected for the losses incidental to the procedure, because to a very large extent the various errors balanced each other and the magnitude of the final correction would have been within the experimental error of reading the colorimeter. The loss due to the removal of the wet paper from the 3 ml. of water in the colorimeter tube before the addition of the thymol blue reagent causes a loss of about 3 % (approximately 0·1 ml. of liquid is removed from a total vol. of 3 ml.) in the total amount of acid present. The removal of this liquid causes the final volume after the addition of the thymol blue reagent to be smaller than if no paper had been added. This increases the colorimeter reading relative to a blank in which no paper had been introduced by 1–2 %. As mentioned previously, the blank readings must be made on paper which has been cut from a chromatogram freshly dried in the absence of carbon dioxide.

Some results on the analysis of made-up mixtures of acids are given in Table 3.

It was found in preliminary experiments that if too great a concentration of the mixture of ammonium salts was applied to the chromatogram, the 'overloading' caused serious errors in the estimation of the individual acids. Extraction of the individual acid spots and re-examination on a paper chromatogram showed that each contained a certain amount of the other acids. In addition, distortion of the shape of the spots which occurred made it difficult to distinguish closely related acids. In the present experiments the acids were added in no greater concentration than 0·3 N. In difficult cases where a small amount of one acid had to be determined in the presence of a large amount of another, it was found best to apply the mixture as a streak to the starting line of the chromatogram. By doing this a large volume of the solution could be examined without increasing the concentration above the safe level. The appropriate strip of paper after development of the chromatogram was extracted and the acid either determined directly on this extract or the concentrated extract re-examined on a paper chromatogram and estimated as described previously.

The recovery of acids added to extracts of cress seedlings (2 mg. of malic and 2 mg. of glyceric acids to 2 g. of cress seedlings) and separated from the bulk of the other solutes by the method of Isherwood (1946) and then estimated using the standard procedure was better than 95 %.

SUMMARY

1. Qualitative examination of a number of common aliphatic acids on a paper chromatogram using *n*-propanol:concentrated aqueous ammonia as solvent, has shown that many of them can be readily separated. In the present paper R_F values are given and the results are also expressed in terms of $\log_{10}\left(\frac{1}{R_F}-1\right)$ plotted against the number of carbon atoms in the molecule in order to throw light on relationships in the chromatographic behaviour of the acids.

2. A quantitative method is described for the estimation of the ammonium salts of the acids after they have been separated on a similar paper chromatogram. It is based on the effect of the ammonium salts on a thymol blue-glycine reagent which is insensitive to free ammonia. The change in colour of the thymol blue is inversely proportional to the concentration of the salt from about 80 % to about 20 % neutralization of the reagent. The accuracy of the method (single determinations) is about ± 10 % when 50 μg. of organic acid is present.

As regards one of us (F.A.I.) the work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

REFERENCES

Brown, F. B. J. (1950). *Biochem. J.* **47**, 598.
Brown, F. B. J. (1951). *Nature, Lond.*, **167**, 441.
Brown, F. B. J. & Hall, L. P. (1950). *Nature, Lond.*, **166**, 66.
Duin, H. V. (1953). *Biochim. biophys. Acta*, **10**, 343.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Hiscox, E. R. & Berridge, N. J. (1950). *Nature, Lond.*, **166**, 522.
Isherwood, F. A. (1946). *Biochem. J.* **40**, 688.
Isherwood, F. A. & Jermyn, M. A. (1951). *Biochem. J.* **48**, 515.
Kennedy, E. P. & Barker, H. A. (1951). *Analyt. Chem.* **23**, 1033.
Long, A. G., Quayle, J. R. & Stedmann, R. J. (1951). *J. chem. Soc.* p. 2197.
Martin, A. J. P. (1949). *Symp. biochem. Soc.* **3**, 4.
Reid, R. L. & Lederer, M. (1952). *Biochem. J.* **50**, 60.

The Combination of some Vitamin B_{12}-like Compounds with Sow's Milk Whey and 'Intrinsic Factor' Concentrates

By MARGARET E. GREGORY and E. S. HOLDSWORTH
National Institute for Research in Dairying, University of Reading

(*Received* 19 *June* 1953)

In a previous communication from this laboratory, Gregory, Ford & Kon (1952) reported the presence in sow's milk of a substance that combined with vitamin B_{12} making it unavailable to assay micro-organisms. This 'binding factor' appeared to be associated with a particular fraction of whey proteins. Other naturally occurring materials have been shown to inactivate vitamin B_{12} in the same way. One of the first reports of this type of inactivation was that of Ternberg & Eakin (1949), who found that normal gastric juice and an aqueous extract of pig gastric mucosa contained a non-dialysable, heat-labile substance that combined with vitamin B_{12} and made it unavailable to assay micro-organisms. Prusoff, Meacham, Heinle & Welch (1950) fractionated extracts of desiccated pig stomach with ammonium sulphate and found that intrinsic factor activity was greatest in the fraction precipitated by 35–55 % saturation with ammonium sulphate. The ability of the various fractions to prevent the utilization of vitamin B_{12} by *Lactobacillus leichmannii* was investigated, but no figures were published in the communication.

The vitamin B_{12}-binding power of proteins such as egg albumin, globulins from blood and from soya beans, urease, lysozyme and an intrinsic factor concentrate has been investigated by Bird & Hoevet (1951). Only the intrinsic factor concentrate, however, showed any appreciable vitamin B_{12}-binding activity. Beerstecher & Altgelt (1951) observed a substance in saliva, similar to Castle's intrinsic factor from gastric juice in its ability to combine with and inactivate vitamin B_{12}. However, these two substances appear to differ in their heat stabilities (Beerstecher & Edmonds, 1951). Using dialysis techniques, in conjunction with microbiological assays, Rosenthal & Sarett (1952) have shown that vitamin B_{12} was present in a bound form in serum and that the serum was also capable of binding limited amounts of added vitamin B_{12}. Chow & Davis (1952) reported that yeast nucleic acid and heparin combined with vitamin B_{12}, but gastric juice was much more effective. Their measurements were made using radioactive vitamin B_{12} and no microbiological assays were carried out.

Compounds are known to exist, such as pseudovitamin B_{12}, first isolated by Pfiffner *et al.* (1951), and factors *A* and *B*, isolated from calf faeces by Ford & Porter (1952), that are not simple derivatives of vitamin B_{12}, but have 'vitamin B_{12} activity' for micro-organisms. Since the combination of vitamin B_{12} with intrinsic factor or other heat-labile substances is thought to be of importance in the metabolism of vitamin B_{12} (Ungley, 1951), it is of interest to find out whether the other vitamin B_{12}-like compounds also form a complex with intrinsic factor, particularly since pseudovitamin B_{12} has been shown to be clinically inactive in the treatment of pernicious anaemia (Pfiffner, Dion & Calkins, 1952). For this reason the microbiological inactivation of pseudovitamin B_{12} and of factors *A* and *B* by an 'intrinsic factor' concentrate and a concentrate from sow's milk has been investigated.

EXPERIMENTAL

Preparation of the sow's whey concentrate. Skimmed sow's milk was clotted with crystalline rennin at 40° and the curd removed. The whey was adjusted to pH 4·6 with glacial acetic acid and the proteins were fractionated by $(NH_4)_2SO_4$ saturation following the procedure used by Prusoff *et al.* (1950) for the concentration of intrinsic factor activity from powdered hog stomach. The proteins precipitated by

33–100% $(NH_4)_2SO_4$ saturation were suspended in a small volume of water, dialysed for 36 hr. against running water and freeze-dried. The freeze-dried proteins were dissolved in 0·1 M sodium acetate buffer at pH 5·5. Saturated $(NH_4)_2SO_4$ solution was added drop-wise with continuous stirring. Precipitates were collected at various concentrations of $(NH_4)_2SO_4$, dissolved in the minimum amount of water, dialysed for 7 hr. against running water and freeze-dried. The fraction precipitated by 66–100% $(NH_4)_2SO_4$ saturation contained the most vitamin B_{12}-binding activity. This was the concentrate used in these experiments. A 1% solution was made in sterile distilled water. From this, serial dilutions were made aseptically for addition to the assay tubes.

Preparation of the 'intrinsic factor' concentrate. The 'intrinsic factor' concentrate was prepared from 'Extomak', a commercial brand of desiccated hog stomach prepared by Benger's Ltd., Holmes Chapel, Cheshire. A saline extract was fractionated by the method described by Prusoff *et al.* (1950). The concentrate used in these experiments was the fraction precipitated by 35–100% $(NH_4)_2SO_4$ saturation, which was dialysed, freeze-dried, reconstituted in a 1% solution in sterile water and diluted aseptically in the same manner as the sow's whey concentrate.

Vitamin B_{12} and vitamin B_{12}-like compounds. The vitamin B_{12} used was the commercially available 'Cytamen' (Glaxo Laboratories Ltd.), which was ionophoretically pure. Factors *A* and *B* and pseudovitamin B_{12} were purified by repeated ionophoresis in 0·5 N acetic acid as previously described (Holdsworth, 1953). Each of the four compounds moved as a single spot on paper chromatograms or on ionophoresis on paper.

Optical density measurements. Since the ionophoretically pure factors were not available in amounts that could be weighed, standard solutions were prepared on a colorimetric basis. The absorption curve of a solution of vitamin B_{12} has a sharp peak at 361 mμ. and at this wavelength the $E^{1\%}_{1\,cm.}$ is 210. Pseudovitamin B_{12} and factor *A* show similar absorption curves to vitamin B_{12}. In the presence of excess of cyanide ion all three substances showed a shift of the absorption peak to 367 mμ. with only a slight change in optical density. Factor *B*, however, had an absorption peak in water at 355 mμ. which shifted to 367 mμ. in 0·01% KCN, and became more sharply defined; and the optical density increased approximately 20% (Fig. 1). Since the absorption peak at 367 mμ. is common to all these substances when tested in the presence of cyanide, colorimetric measurements were made at this wavelength in 0·01% KCN. The measurements were made with the Beckman spectrophotometer and the concentrations calculated on the assumption that the $E^{1\%}_{1\,cm.}$ for pseudovitamin B_{12} and factors *A* and *B* was the same as for vitamin B_{12}. On this basis the factors were diluted to a concentration of 1 μg./ml. with 20% (v/v) aqueous ethanol containing 0·01% (w/v) KCN, and stored at 1°.

Measurement of binding activities. The vitamin B_{12}-binding activity of the sow's whey or 'intrinsic factor' concentrates was measured by adding decreasing amounts of the concentrates to a fixed amount of vitamin B_{12}. As the amount of 'binding material' in the tubes decreased so the microbiological availability of the vitamin increased. Where the amount of 'binding material' in the tube was such that it completely 'bound' all the vitamin B_{12}, the assay organism was unable to grow. But where the quantity of 'binding material' present was only sufficient to inactivate a part of the vitamin B_{12} the assay organism responded to the residual 'unbound' vitamin. Quantitative measurement of this response by reference to a standard containing vitamin B_{12} alone allowed the calculation of the amount of the vitamin 'bound' by 1 g. of the freeze-dried concentrate (see Table 1).

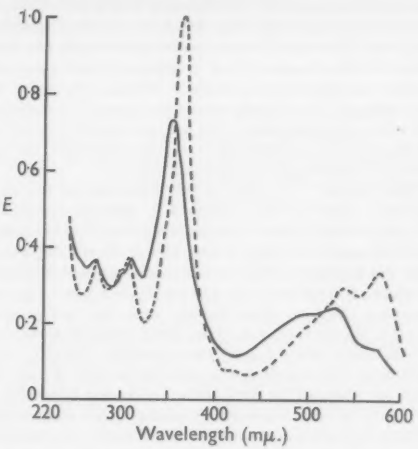


Fig. 1. Absorption spectra of factor *B* (solid line) and its cyano adduct (broken line).

Table 1. *Estimation of the vitamin B_{12}-binding activity of sow's whey concentrate*

(Assay organism: *Escherichia coli*. Vitamin B_{12} (0·4 μmg.) was added to each tube.)

Sow's whey concentrate/ tube (μg.)	Vitamin B_{12} available to *Esch. coli* (μmg.)	Vitamin B_{12} 'bound'/tube (μmg.)	Vitamin B_{12} 'bound'/g. concentrate (μg.)
5·000	0·00	>0·4	>80
2·500	0·15	0·25	100
1·250	0·28	0·12	96
0·625	0·34	0·06	96

The amounts of factors *A* and *B* and pseudovitamin B_{12} 'bound' were calculated in the same way by reference to a standard of the particular compound under test.

Microbiological methods. The method of assay was based on the *Escherichia coli* tube assay described by Burkholder (1951), except that the sodium thioglycollate in the medium was replaced by thiomalic acid. The medium was made up at 5 times its single strength, and 1 ml. added to each tube making the final volume of liquid to 5 ml., as described below.

A standard of vitamin B_{12} was set up by preparing a solution containing 0·2 μmg./ml. and adding it to a series of assay tubes at levels of 0·2, 0·5, 1·0, 1·5, 2·0, 3·0 and 4·0 ml. per tube, i.e. over a range of 0·04–0·8 μmg. per tube. The volume of liquid in each tube was made up to 4 ml. with distilled water and 1 ml. of medium added.

For the measurement of 'binding activity' a series of tubes, containing the assay medium plus 0·4 μmg. vitamin B_{12} in a total volume of 4 ml. was sterilized by steaming for 30 min. After cooling, aseptic additions of 1 ml. portions of

the serial dilutions of the concentrates were made. A similar set of tubes was prepared in which the 'binding material' was added before sterilization to obtain a value for the heat-stable 'binding activity'. A preliminary trial assay was made to ascertain the approximate 'binding activity' of the concentrates. The dilutions were then arranged so that they fell within a narrow range, thus enabling two or three of the dilutions to give growth responses from which the 'binding activity' could be calculated (see Table 1).

The 'binding' of factor *A* was measured in the same manner, using as the standard a solution containing 0·2 μmg. of factor *A*/ml. (colorimeter potency). The method was the same for factor *B* and pseudovitamin B_{12} except that the standard solutions were made, respectively, 5 and 10 times stronger than the vitamin B_{12} solution. This was necessary because these factors were less active than vitamin B_{12} for *Esch. coli*, when their concentrations were calculated on optical density measurements. The tubes were inoculated with one drop of a saline suspension of *Esch. coli* cells, as described by Burkholder (1951) and incubated at 30° for 18 hr. with continuous shaking. After steaming for 10 min., the tubes were cooled, 5 ml. of water were added to each (making a total volume of 10 ml.) and the growth was measured turbidimetrically.

RESULTS

Factors *A* and *B*, and pseudovitamin B_{12}, were less active than vitamin B_{12} in promoting the growth of *Esch. coli*. For example, a solution of factor *A* containing 1 μg./ml. by colorimetric measurement had growth activity for *Esch. coli* equivalent to only 0·66 μg. vitamin B_{12}/ml. These activities have been called the 'vitamin B_{12} activities' of the compounds, since they are a measure of the growth-promoting activity of these compounds for *Esch. coli* in terms of vitamin B_{12}. In Table 2 they are compared with the colorimetric potencies. It follows that to obtain the same amount of growth as given by 1 μmg. vitamin B_{12}, 1·33 μmg. factor *A*, 4·5 μmg. factor *B* and 12 μmg. pseudovitamin B_{12} by colour potency are required.

The amounts of vitamin B_{12} and vitamin B_{12}-like compounds 'bound' by the 'intrinsic factor' and sow's whey concentrates are given in Table 3. These figures were calculated from the concentrations of each of the compounds measured colorimetrically as described above. Because of the different growth activities of the compounds for *Esch. coli*, their concentrations had to be chosen so that they gave approximately the same amount of growth as the standard vitamin B_{12} solution, which contained 0·2 μmg./ml. Consequently solutions at concentrations of 0·2 μmg./ml. for factor *A*, 1·0 μmg./ml. for factor *B* and 2 μmg./ml. for pseudovitamin B_{12} were used. This necessitated the use of stronger solutions of the 'binding' concentrates for factor *B* and pseudovitamin B_{12}. In spite of these differences in the way the 'binding activities' were measured, the amounts of the vitamin B_{12}-like compounds 'bound' by the concentrates were remarkably constant.

The 'vitamin B_{12} activities' were also used for calculating the amounts of the compounds 'bound'

Table 2. *The 'vitamin B_{12} activities' of factor* A, *factor* B *and pseudovitamin B_{12} measured by the* Escherichia coli *tube assay*

(The concentration of all compounds was 1 μg./ml. as estimated by colorimetric measurement. The number of determinations is given in parentheses.)

Vitamin B_{12}-like factor	'Vitamin B_{12} activity' (±standard error) (μg./ml.)
Vitamin B_{12}	1·00
Factor *A*	0·58±0·09 (4)
Factor *B*	0·18±0·04 (5)
Pseudovitamin B_{12}	0·08±0·02 (3)

Table 3. *Amounts of vitamin B_{12}, factors* A *and* B *and pseudovitamin B_{12} 'bound' by 'intrinsic factor' and sow's whey concentrates*

(Calculated from the concentrations of each compound measured colorimetrically. Assay organism: *Esch. coli*. Individual experimental results are given with their mean. The result of each experiment was calculated from three determinations at different levels of the concentrate as shown in Table 1.)

		Vitamin B_{12} 'bound' (μg./g.)	Factor *A* 'bound' (μg./g.)	Factor *B* 'bound'* (μg./g.)	Pseudovitamin B_{12} 'bound' (μg./g.)
			'Intrinsic factor' concentrate		
Heated		13, 14, 10, 8	8, 12, 6, 7	11, 2, 2	14, 27, 13
	Mean	**11**	**8**	**5**	**18**
Unheated		190, 200, 210, 170	140, 150, 170, 130	120, 150, 160	150, 230, 220
	Mean	**190**	**150**	**140**	**200**
			Sow's whey concentrate		
Heated		30, 12, 15	12, 6, 9	4, 3	21, 13
	Mean	**19**	**9**	**3·5**	**17**
Unheated		110, 120, 100	100, 80, 100	90, 130	130, 110
	Mean	**110**	**93**	**110**	**120**

* Values were reduced by 20% if the colour was measured at 355 mμ. in the absence of cyanide.

by the concentrates. The results are not given in detail since it was found that, calculated in this way, the amounts of factor *A*, factor *B* and pseudovitamin B_{12} 'bound' were 66, 22 and 8 %, respectively, of the figures given in Table 3. That is, they merely reflected the growth-promoting activities of these compounds for *Esch. coli* (see Table 2).

DISCUSSION

The ideal way of calculating the amounts of the vitamin B_{12}-like compounds which combine with 'intrinsic factor' and sow's whey concentrates would be on an equimolecular basis. But, since the chemical constitution of these compounds is still unknown, this is not possible at present. By measuring the absorption of solutions of these compounds in the region 350–370 mμ. we have assumed that a functional part of the molecule, common to all of the vitamin B_{12}-like compounds tested, was being measured. The results based on these measurements (Table 3) seem to justify this assumption, since vitamin B_{12} and the other compounds are all 'bound' to the same extent.

As a temporary expedient, therefore, it may be useful to base microbiological assays of these vitamin B_{12}-like compounds on colorimetric concentrations, measured as described in this paper. We are well aware of the possible fallacy in our assumption that the $E_{max.}$ for a compound closely related to vitamin B_{12} is necessarily the same as for vitamin B_{12}. The presence or absence of a particular grouping could sharpen or depress the absorption peak. With pseudovitamin B_{12}, in which the dimethylbenziminazole nucleotide moiety of vitamin B_{12} is replaced by adenylic acid (Dion, Calkins & Pfiffner, 1952), the $E_{max.}$ is, however, the same as for vitamin B_{12}. This is also the case with factor *A*, although it is not yet known how this compound differs from vitamin B_{12}.

Factor *B* is a less complex molecule than vitamin B_{12}, but its constitution is not yet known; it has $E_{max.}$ at 355 mμ., but when a slight excess of cyanide is added the peak becomes sharper and shifts to 367 mμ. The concentrations of factor *B*, calculated from the absorption at 355 mμ. without cyanide and at 367 mμ. in the presence of cyanide, were both used for estimating the 'binding' of factor *B* by the concentrates. The results obtained in the absence of cyanide were 20 % less than those given in Table 3. Since the absorption peak of the vitamin B_{12}-like compounds at 367 mμ. in the presence of cyanide seemed to be common to all, the results in Table 3 were calculated using this value. It seems probable that absorption in the region 367 mμ. is due to a part of the molecule to which the protein concentrates become attached and which is common to all the factors.

In our microbiological assays we have always been able to measure some form of 'binding activity' that was stable to steaming for 30 min. The original report of sow's milk 'binding activity' (Gregory *et al.* 1952) referred to this heat-stable binding component. Table 3 shows that the unheated concentrates 'bind' many times more vitamin B_{12} (or vitamin B_{12}-like compounds) than the heated concentrates. Since the completion of the above experiments, however, we have found that the heat stability of the binding substances depended on the particular reducing agent used in the medium. Substitution of ascorbic acid for thiomalic acid increased stability considerably, so that the binding activity, measured after heating at 100° for 30 min. in medium containing ascorbic acid, was about half the unheated activity.

In a review of the work done on the 'intrinsic factor', Welch & Nichol (1952) stress the fact that the term 'intrinsic factor' should not be used synonymously with 'vitamin B_{12}-binding substances'. The 'intrinsic factor' concentrate used in these experiments was not tested for its clinical activity, nor was the sow's whey concentrate. However, Latner, Ungley, Cox, McEvoy-Bowe & Raine (1953) have fractionated human gastric juice by electrophoresis and found two peaks, one moving to the anode and the other to the cathode, which possessed both intrinsic factor and vitamin B_{12}-binding activities. Examination of our concentrates by electrophoresis showed only one zone with vitamin B_{12}-binding activity moving towards the anode with similar mobility to Latner's peak. Only a small proportion of the total solids of our concentrates was present in the zone of binding activity. In the case of the sow's whey concentrate it was 5 % of the total protein present. Further work on the isolation and purification of the binding substance from sow's whey will be published elsewhere.

If combination with intrinsic factor is important for the absorption of vitamin B_{12} or serves to protect it from assimilation by the intestinal flora, then the same process would apply to the other vitamin B_{12}-like compounds, since they are 'bound' to the same extent as vitamin B_{12}. It follows then that the clinical inactivity of the compounds other than vitamin B_{12}, e.g. pseudovitamin B_{12} (Pfiffner *et al.* 1952), is not due to their inability to combine with intrinsic factor.

SUMMARY

1. Optical density measurements have been used to determine the concentrations of solutions of ionophoretically pure vitamin B_{12}, factors *A* and *B* and pseudovitamin B_{12}.

2. From these measurements the microbiological activities for *Bact. coli*, in a tube assay, have been established. Factor *A* had 66 %, factor *B* 22 % and

pseudovitamin B_{12} 8% of the growth activity of vitamin B_{12}.

3. The amounts of these factors which combined with an 'intrinsic factor' concentrate and a sow's whey concentrate were identical with the amount of vitamin B_{12} 'bound'.

The authors wish to express their thanks to Dr N. J. Berridge for the crystalline rennin used in the preparation of the sow's whey concentrate, to Dr R. Braude and Dr K. G. Mitchell for the samples of sow's milk, to Dr E. Lester Smith for gifts of factor *B* and to Dr S. K. Kon for his interest and suggestions throughout this work.

Note. Since this paper was written, J. B. Armitage *et al.* (*J. chem. Soc.* in the Press) have identified factor *B* as vitamin B_{12} less the dimethylbenzaminazole nucleotide portion.

REFERENCES

Beerstecher, E. jun. & Altgelt, S. (1951). *J. biol. Chem.* **189**, 31.
Beerstecher, E. jun. & Edmonds, E. J. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 563.
Bird, O. D. & Hoevet, B. (1951). *J. biol. Chem.* **190**, 181.
Burkholder, P. R. (1951). *Science*, **114**, 459.
Chow, B. F. & Davis, R. L. (1952). *Nutrition Symposium Series*, no. 5, p. 17. N.Y. The National Vitamin Foundation Inc.
Dion, H. W., Calkins, D. G. & Pfiffner, J. J. (1952). *J. Amer. chem. Soc.* **74**, 1108.
Ford, J. E. & Porter, J. W. G. (1952). *Biochem. J.* **51**, v.
Gregory, M. E., Ford, J. E. & Kon, S. K. (1952). *Biochem. J.* **51**, xxix.
Holdsworth, E. S. (1953). *Nature, Lond.*, **171**, 148.
Latner, A. L., Ungley, C. C., Cox, E. V., McEvoy-Bowe, E. & Raine, L. (1953). *Brit. med. J.* **1**, 467.
Pfiffner, J. J., Calkins, D. J., Peterson, R. G., Bird, O. D., McGlohon, V. & Stipek, R. W. (1951). *Abstr. Pap. Amer. Chem. Soc.* 120th Mtg, p. 226.
Pfiffner, J. J., Dion, H. W. & Calkins, D. G. (1952). *Fed. Proc.* **11**, 269.
Prusoff, W. H., Meacham, G. C., Heinle, R. W. & Welch, A. D. (1950). *Abstr. Pap. Amer. Chem. Soc.* 118th Mtg, p. 27A.
Rosenthal, H. L. & Sarett, H. P. (1952). *J. biol. Chem.* **199**, 433.
Ternberg, J. L. & Eakin, R. E. (1949). *J. Amer. chem. Soc.* **71**, 3858.
Ungley, C. C. (1951). *Nutr. Abstr. Rev.* **21**, 1.
Welch, A. D. & Nichol, C. A. (1952). *Annu. Rev. Biochem.* **21**, 652.

The Pigments in Colour Phases of the Larvae of *Plusia gamma* L. (the Silver-Y Moth)

By T. W. GOODWIN
Department of Biochemistry, The University Liverpool

(*Received* 6 *June* 1953)

Uvarov first proposed his theory of phases in 1921 (see Uvarov, 1928), one of the characteristics of insects in the 'gregarious' phase being that they are much darker in colour than those in the 'solitary phase'. The biochemical changes accompanying the colour variations in gregarious and solitary phases of *Locusta migratoria* and *Schistocerca gregaria* (order Orthoptera) have recently been investigated (see Goodwin, 1952*a*). Phases in the larvae of Lepidoptera were first observed by Faure in South Africa (1943*a*, *b*), namely, *Laphygma exigua* (the lesser army worm) and *L. exempta* (the army worm); they also probably exist in *Spodoptera abyssinia*.

Colour variations have recently been noted in this country between crowded and solitary larvae of the lepidopteran *Plusia gamma* both reared from the same batch of eggs. The solitary larvae remained pale green with thin white longitudinal stripes, whilst the crowded larvae showed forms ranging from a similar colour to a much darker green with yellow stripes. This variation was also observed in the field where unusually dark larvae were associated with mass outbreaks (Williams & Long, 1950).

This paper describes an investigation into the pigments responsible for the colour changes in *Plusia gamma*.

EXPERIMENTAL

Rearing of larvae. The larvae were reared at Rothamsted Experimental Station on sprigs of agricultural mustard stood in water in 1000 ml. short beakers fitted with fine muslin covers. A pad of filter paper in the bottom of each beaker prevented excessive dampness and provided a suitable surface for the larvae.

A few hours after hatching the young larvae from each batch of eggs were distributed at random over the breeding jars to provide both crowded cultures of eighty larvae and solitary cultures. Under these conditions the solitary larvae become a light green, whilst the vast majority of those in crowded cultures become a dark olive green.

Throughout the period of development a surplus of food was maintained in all the breeding cultures. Larvae in the middle of their last instar were then selected from the two conditions of culture and sent alive to Liverpool for examination.

Examination of haemolymph. The larvae were killed with ether and the cuticle was opened at the posterior end. The haemolymph which was squeezed out was bright green. It was collected either directly into a small test tube or, if required for spectroscopic analysis, into a dilute solution (0·1%, w/v) of KCN; this inhibited tyrosinase activity. The collected haemolymph was examined for carotenoproteins, after direct extraction by light petroleum (light petroleum, b.p. 40–60° was used throughout this investigation) had failed to reveal the presence of free carotenoids (Goodwin & Srisukh, 1949). An equal volume of ethanol was added to the lymph and the pigments thus liberated by the denaturation of the proteins were extracted from the aqueous phase with light petroleum. The extinction of the extract was measured and used to determine the amount of carotenoids present, calculated as lutein and using $E^{1\%}_{1\,cm.} = 2500$ for pure lutein (Zscheile, White, Beadle & Roach, 1942).

The aqueous residue was tested for bile pigments by extracting with ethyl acetate after acidification with HCl. The amount of bile pigment present was determined by measuring the extinction of the extract at 670 mμ. and assuming $E^{1\%}_{1\,cm.}$ for pure mesobiliverdin to be 527 (Lemberg & Legge, 1949).

Examination of the integument. The integument, freed from all internal organs, was examined for carotenoids in a number of ways. (*a*) The tissues were ground with anhydrous Na_2SO_4 and acid-washed silver sand until a fine powder was obtained; this powder was then extracted with successive portions of diethyl ether (freshly distilled from reduced iron) until all the pigment was removed. The ether was removed *in vacuo* and the residue made up to a known volume with light petroleum. This was then used for chromatographic examination after making an extinction reading at 445 mμ. in order to determine the total amount of carotenoids present. (*b*) The integument was ground with 0·067 M potassium phosphate buffer (pH 7·0) and silver sand and centrifuged; the supernatant, which would contain any carotenoproteins present, was treated in the same way as haemolymph. The residue which contained the remaining carotenoids was examined as described under (*a*). (*c*) The integument was ground with 0·2 N-NaOH and silver sand; after centrifugation, the supernatant, which contained much more material than the pH 7 extract, was treated with glacial acetic acid until just acid. A copious precipitate was produced which, when collected by centrifugation, was examined for carotenoids as described under (*a*).

In each method the material remaining after removal of carotenoids was extracted with either ethyl acetate acidified with HCl to extract any bile pigment present or with methanol containing 5% (v/v) 10 N aqueous HCl, which would remove any insectorubin (Goodwin & Srisukh, 1950).

Examination of carotenoids present. Chromatographic separation and purification of the carotenoid pigments were carried out on columns either of alumina (P. Spence, Ltd., Widnes, Grade 'O') weakened according to the method of Goodwin & Srisukh (1949) or $CaCO_3$ of analytical grade. Ordinary laboratory $CaCO_3$ is not suitable as it may contain considerable amounts of $Ca(OH)_2$ (Lederer, 1952).

Lutein (3:3′-dihydroxy-α-carotene) which was required as a reference carotenoid, was obtained from two sources, grass and leaves of tomato plants. This pigment was also purified by chromatography using the above-mentioned adsorbents.

Examination of bile pigment present. The tests for mesobiliverdin described by Goodwin & Srisukh (1951) were carried out.

RESULTS

Haemolymph

Specimens of haemolymph from both solitary and crowded *P. gamma* larvae were always bright green and did not differ significantly in their absorption spectra; a typical curve obtained by diluting haemolymph with 0·1% (w/v) potassium cyanide is given in Fig. 1.

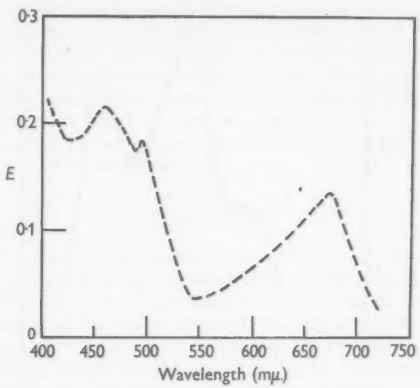


Fig. 1. The absorption spectrum of haemolymph from gregarious *P. gamma* larvae. The haemolymph was diluted with 0·1% (w/v) KCN; the exact dilution was not known but was of the order 1:10.

Carotenoids. As little or no carotenoid material was directly extractable from haemolymph with light petroleum, it appeared that, as in the case of the haemolymph of other insect species so far examined, the pigments were conjugated with protein. The haemolymph proteins were therefore denatured with ethanol, after which the carotenoids were easily extractable with light petroleum. The light-petroleum fraction was chromatographed on weakened alumina. No β-carotene was observed, thus confirming preliminary partition experiments which suggested that the pigments were entirely free xanthophylls; on elution with light petroleum containing 50% (v/v) diethyl ether, a major yellowish brown zone (A) moved slowly down the column, leaving behind a lemon-yellow band (B) which travelled down more slowly.

After purification of fraction A by chromatography on calcium carbonate, the spectrum (λ_{max} 420, 445 and 470 mμ. in light petroleum) indicated that it was lutein. This was confirmed (*a*) by com-

parison with the spectrum of an authentic specimen of lutein measured in three solvents, namely, ethanol, benzene and light petroleum and (*b*) by demonstrating that on chromatography on alumina and on calcium carbonate a mixture of the haemolymph pigment and authentic lutein did not separate. The absorption spectrum of pigment A and of lutein is shown in Fig. 2.

Fraction B only occurred in small amounts (about 10 %, at most, of the total carotenoids) and was not identified; its spectrum ($\lambda_{max.}$ 419, 441, 469 mμ. in light petroleum), and its adsorptive properties suggest that it might possibly be lutein-5:6-epoxide.

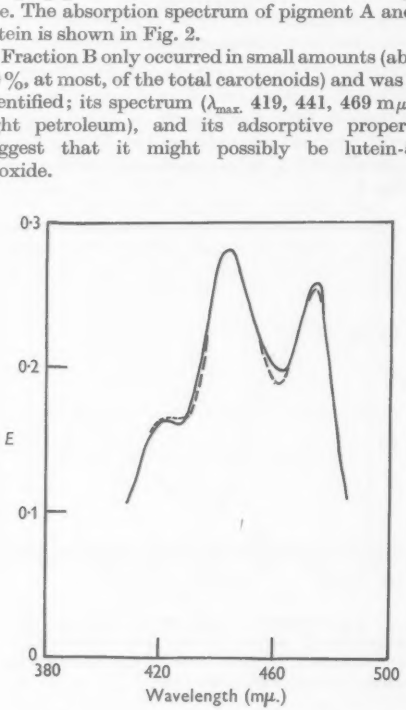


Fig. 2. The absorption spectra in light petroleum (b.p. 40–60°) of lutein and the major carotenoid from the haemolymph of *P. gamma* larvae. The *E* values of the two pigments were adjusted so as to coincide at the main peak. – – –, authentic lutein; ——, haemolymph pigment. The dotted line is shown only when it does not coincide with that of the haemolymph pigment.

Quantitative experiments showed that the concentration of lutein in the haemolymph was high and that there was no difference between solitary and crowded insects reared under otherwise identical conditions (Table 1).

Bile pigments. After removing the carotenoids, the aqueous residue was acidified with hydrochloric acid and extracted three times with ethyl acetate until all colour was removed. Except in the case of the first ethyl acetate extract of one specimen of haemolymph, which was blue, all the extracts were bluish green. It was concluded that this pigment was probably mesobiliverdin for the following reasons: (*a*) it exhibited an absorption maximum 665 mμ. (670 mμ.) in acidified solvents (methanol); (*b*) it formed a green zinc complex, $\lambda_{max.}$ 675 mμ. (685 mμ.), with no fluorescence in ultraviolet light and which on addition of iodine shifted its absorption maximum to 629 mμ. (630 mμ.) and fluoresced pink. (The absorption maxima in parentheses are those recorded for authentic mesobiliverdin by Lemberg & Legge, 1949.) Mesobiliverdin is already known to exist in the haemolymph of other insect species (Junge, 1941; Goodwin & Srisukh, 1951; Hackman, 1952).

The blue extract encountered on one occasion had its absorption maximum at 636 mμ. and not 665 mμ. The blue pigment which Goodwin & Srisukh (1951) obtained from locust haemolymph exhibited all the properties of mesobiliverdin except that the absorption-spectrum maximum in acid methanol was shifted from 670 to 647 mμ. The reason for the single appearance of this type of spectrum in one *P. gamma* extract (the remaining ethyl acetate extracts of the same haemolymph were bluish green) is not known. The amounts of mesobiliverdin in the haemolymph of solitary and crowded *P. gamma* are recorded in Table 1; there are obviously no marked differences between the mesobiliverdin content of the two colour phases.

Integument

The carotenoid extract was chromatographed on weakened alumina. On developing with light petroleum containing 50 % (w/v) diethyl ether the

Table 1. *Pigment distribution in solitary and gregarious larvae of* Plusia gamma

(The figures in brackets indicate the numbers of skins examined in each experiment.)

	Integument (mg./skin)		Haemolymph (mg./100 ml.)	
	Solitary	Gregarious	Solitary	Gregarious
Total carotenoids (measured as lutein)	1·56 (11) 1·20 (13)	2·09 (45) 1·60 (64)	2·04 1·61 1·81	— 1·63 —
Mesobiliverdin	—	0·0012* (64)	3·9 2·3 4·0 .	2·3 — —
Melanin	Absent	Present	Absent	Absent

* May be due to contamination of integument with haemolymph.

chromatogram described in Table 2 was obtained. Previously it had been noted that, at the beginning of the experiment, development with light petroleum containing 1% (v/v) diethyl ether removed a very small trace of pigment from the column; it could not be completely identified but was in all probability β-carotene. Fraction A, containing the bulk of the pigment (at least 90%) was eluted with ethanol, purified by chromatography on calcium carbonate and proved to be lutein by the criteria described for the haemolymph pigment. Fraction B, which occurred only in traces, had a spectrum very similar to lutein and was perhaps a *cis*-isomer of lutein. The greenish yellow band C was not completely identified, but from its absorption spectrum and its position on the column appears to be identical with the haemolymph pigment B. If the integument residue remaining after the removal of carotenoids is extracted with acidified ethyl acetate then traces of a pigment are obtained exhibiting an absorption maximum at 665 mμ. It must be concluded that the pigment is probably mesobiliverdin, although it is not possible to say whether it exists as such in the skin or was extracted from small amounts of haemolymph remaining adsorbed on the skin. This latter possibility is quite feasible, for one quantitative determination indicated the presence of about 1 μg. of mesobiliverdin in one skin; this could easily have come from slight contamination with haemolymph containing about 20 μg./ml.

Table 2. *The separation of the carotenoids of the integument of the larvae of* Plusia gamma

(Adsorbent, weakened Al_2O_3; developer, light petroleum containing 50% (v/v) diethyl ether. The zones are listed in order of increasing adsorptive power.)

Zone	Description	Absorption spectrum maxima (mμ.) (light petroleum)
A	Yellow-orange: major zone	446, 471
B	Brownish orange: small	444, 470
C	Lemon-yellow	441, 469

An aqueous extraction at pH 7 of the finely ground integument yielded only traces of combined lutein, whilst an extract made with 0·2N sodium hydroxide contained somewhat more. This shows that the major portion of the skin lutein is not in the form of a carotenoprotein. The amounts of lutein present in the skins of crowded and solitary *P. gamma* are given in Table 1.

Extraction of the skin residue with methanol containing 5% (v/v) 10N aqueous hydrochloric acid after removal of the carotenoids yielded a colourless solution, thus indicating that no insectorubin was present.

After extraction with the above solvents the remaining skin residue from crowded insects contained black patches; these were assumed to be due to the presence of melanin. Similar residues of skins of solitary larvae indicated the absence of melanin, for no black patches were observed. These findings are in accordance with those of Long (1953) who made both histological and microscopical examinations of larval integuments. Although melanin is absent from the integument of solitary specimens the melanin-producing enzyme tyrosinase is not. Aqueous extracts of the skin quickly go black on standing, as do the extracts from crowded larvae; this blackening is inhibited by potassium cyanide for both crowded and solitary larvae. Tyrosinase activity of haemolymph from both forms of larvae is also indicated by blackening on standing, which can be inhibited by cyanide. Although no precise measurements were made, it was obvious that haemolymph from solitary larvae took much longer (about 30 min.) to darken than did that from crowded larvae (which was black within 2 min. of removal from the insects).

DISCUSSION

The green pigment of the haemolymph of *P. gamma* is another example of the widely distributed composite pigment 'insectoverdin'. The major component of the yellow fraction is lutein as in *Sphinx ligustri*, *Tettigonia viridissima* and *Meconema* (Junge, 1941), *Pieris rapae* and *Cacoecia australania* (Hackman, 1952), while the blue component is mesobiliverdin as in all other insects so far found to produce insectoverdin. Although the bile pigment does not appear to alter from species to species, the carotenoid component can vary, e.g. in *Dixippus morusus* (Junge, 1941), *Locusta migratoria migratorioides* and *Schistocerca gregaria* (Goodwin & Srisukh, 1951) it is β-carotene and not lutein.

The lutein content of the haemolymph of *P. gamma* adds further evidence to support the generalization that the carotenoid levels in insect blood are very much higher than in the blood of most other animals which contain carotenoids (Palmer & Knight, 1924; Goodwin & Srisukh, 1951; Hackman, 1952), being rivalled only by that of certain breeds of cows grazing on very lush pasture (Goodwin, 1952*b*).

The reason for the preferential accumulation of either lutein or β-carotene (occasionally both can occur together but one always predominates (Hackman, 1952)) by different species of insects is not exactly known but two main possibilities exist: (*a*) one pigment is absorbed from the gut, whilst the other is for the most part excreted, or (*b*) both are absorbed but one is oxidatively destroyed to a much greater extent than the other.

The coloration of P. gamma

The overall light-green coloration of solitary specimens is almost entirely due to the bright green of the haemolymph; if this is squeezed out of an insect then the skin becomes more or less transparent, the white longitudinal stripe remaining translucent. The carotene present in the skin (and the small amounts of mesobiliverdin also possibly present) does not appear to play any major part in the coloration of the larvae. This is different from the case of solitary locusts, the green colour of which is due to the presence of an insectoverdin in the integument (Goodwin, 1952*b*).

Long (1953) found a number of biological similarities to exist between the phases of locusts and the colour phases of *P. gamma* and concluded that the biological principles involved in the production of these phases were the same. It is interesting in this respect to compare the present observations with previous investigations on locusts (Goodwin, 1952*b*).

The darker colour of crowded larvae of *P. gamma* is due to the presence of melanin in the cuticle. This, superimposed on the green of the haemolymph, produces an insect with a much darker-green aspect than the solitary type. As in locusts, the laying down of melanin in the integument of *P. gamma* is a characteristic of the crowded phase. In locusts, however, the production of insectoverdin is inhibited in crowded insects; this is not so in *P. gamma*. A further similarity between locusts and *P. gamma* is that, although melanin is not produced under solitary conditions, tyrosinase activity is not absent. An investigation into the mechanism by which melanin inhibition in the solitary phase is controlled would obviously be of prime importance in the study of the fundamentals of the phase transformations.

The narrow longitudinal line, which is white in solitary *P. gamma*, is yellow in the crowded insects. When pieces of integument containing this region are placed in acetone, the colour is rapidly removed, indicating that it is probably due to a carotenoid. The extracted tissues always show a very slight pink tint at the edge of the stripe; it is possible that this is due to the presence of minute traces of insectorubin. This would originally be present as a brown chromoprotein as in locusts (Goodwin, 1952*b*) and be denatured by the acetone. If this is so, the amounts of insectorubin present are so small as not to be detected by our normal extraction procedures.

SUMMARY

1. The green pigment in the haemolymph of the larvae of *Plusia gamma* is a typical insectoverdin, the yellow component being lutein and the blue component mesobiliverdin.

2. This pigment complex exists in the haemolymph of both the pale-green, solitary, and the dark, crowded forms of *P. gamma*.

3. The integument of both colour phases contains lutein, mostly unattached to protein. Traces of mesobiliverdin may also be present, but insectorubin appears to be absent.

4. The integument of dark, crowded larvae contains melanin, whilst that of the solitary form does not. Tyrosinase activity, however, is present in the haemolymph and integument of both crowded and solitary forms.

5. The quantitative distribution of these pigments in solitary and crowded larvae is recorded.

I am extremely grateful to Dr D. B. Long (Department of Entomology, Rothamsted Experimental Station) for bringing this problem to my notice, supplying the larvae, and allowing me access before publication (Long, 1953) to the results of his investigations on the effects of population density on the morphology, physiology and behaviour of *P. gamma* larvae. Thanks are also due to the Medical Research Council for a grant towards laboratory expenses, and to Prof. R. A. Morton, F.R.S., for his interest in this work.

REFERENCES

Faure, J. C. (1943*a*). *Fmg in S. Afr.* **18**, 69–78.
Faure, J. C. (1943*b*). *Sci. Bull. Dep. Agric. S. Afr.* no. 234.
Goodwin, T. W. (1952*a*). *Biol. Rev.* **27**, 439.
Goodwin, T. W. (1952*b*). *The Comparative Biochemistry of the Carotenoids*. London: Chapman and Hall.
Goodwin, T. W. & Srisukh, S. (1949). *Biochem. J.* **45**, 263.
Goodwin, T. W. & Srisukh, S. (1950). *Biochem. J.* **47**, 549.
Goodwin, T. W. & Srisukh, S. (1951). *Biochem. J.* **48**, 199.
Hackman, R. H. (1952). *Arch. Biochem. Biophys.* **41**, 166.
Junge, H. (1941). *Hoppe-Seyl. Z.* **268**, 179.
Lederer, E. (1952). *Biochim. biophys. Acta*, **9**, 92.
Lemberg, R. & Legge, J. W. (1949). *Haematin compounds and bile pigments, their constitution, metabolism and function*. New York: Interscience.
Long, D. B. (1953). *Trans. R. ent. Soc. Lond.* December.
Palmer, L. S. & Knight, H. H. (1924). *J. biol. Chem.* **59**, 443.
Uvarov, B. P. (1928). *Locusts and Grasshoppers. A Handbook for their Study and Control*. London: Imperial Bureau of Entomology.
Williams, C. B. & Long, D. B. (1950). *Nature, Lond.*, **166**, 1035.
Zscheile, F. P., White, J. W., Beadle, B. W. & Roach, J. R. (1942). *Plant Physiol.* **17**, 331.

The Fungicidal Activity of Substituted Acetanilides and Related Compounds

By N. G. CLARK and A. F. HAMS
Research Department, Boots Pure Drug Co. Ltd., Nottingham

(*Received* 18 *April* 1953)

During a programme of research on the preparation and testing of possible non-metallic organic fungicides, consideration was given to halogenoacylamides, a class of compounds which has been reported in a patent (Maier-Bode, 1940) to possess the desired biological properties. Since few details are given in this publication, which records some of the specific compounds claimed to have fungicidal activity, a detailed examination of this type of compound was of interest and is described in the present paper.

The compounds tested are all *N*-arylamides, the type of compound which is quoted almost exclusively in the patent. An examination of the *N*-alkylamides was reserved for possible future study because three publications (Weaver & Whaley, 1947*a*, *b*; Swensen & Weaver, 1948) describing the preparation of groups of these compounds as potential fungicides suggested that their examination had already been undertaken by other workers. Indeed, this proved to be the case when a later publication (Leonard & Blackford, 1949) announced the relevant biological testing results. Amongst the compounds tested by Leonard & Blackford (1949) are eleven acetanilide derivatives, and the fungicidal activities which they report are compared below with those obtained by the present authors.

EXPERIMENTAL

Preparation of compounds

The compounds selected for study were, with four exceptions, acetanilide derivatives of the general formula

in which R was usually chlorine (though other monovalent groups were sometimes involved), R′ was hydrogen or an *n*-alkyl radical, and in which the benzene ring also carried suitable substituents; the remaining compounds were closely related structurally. Those substances already recorded in the literature were prepared by the methods therein reported or by method *A* (below), whichever were deemed the more suitable. Most of the new acetanilide derivatives were prepared by one of the five general procedures indicated, and the relevant data concerning the yield, physical characteristics and analysis of individual compounds are summarized in Table 1. The preparation and properties of other new substances are recorded separately. Some improved syntheses of known materials are also included. (All melting points are uncorrected.)

Method A. This process is essentially that of Jacobs & Heidelberger (1917). A solution or fine suspension of the amine (10 g. or 10 ml.) in a mixture of glacial acetic acid (50 ml.) and saturated aqueous sodium acetate (50 ml.) was stirred vigorously in a cold-water bath while the requisite acyl chloride (1·2 mol. prop.) was added during 30 min. Generally, the crude product separated directly from the reaction mixture either as an oil or as a crystalline solid. The mixture was diluted with water and any solid was collected, washed with water and recrystallized from ethanol or aqueous ethanol. When the product was an oil, the diluted reaction mixture was extracted with ether, the extract washed with aqueous Na_2CO_3 and water, and then fractionally distilled. *Trichloroacet-2-chloroanilide* (15) was isolated as a viscous oil which solidified after keeping at 0° for 3 weeks.

Method B. A solution of the amine (10 g.) in dry benzene (50–100 ml.) was carefully treated with chloroacetyl chloride (2–5 mol. prop.) in dry benzene (10–20 ml.) and the mixture refluxed for 1–2 hr. on a steam bath. The solvent and excess of chloroacetyl chloride were removed by distillation *in vacuo*, the solid residue was then triturated in very dilute HCl and, after washing with water, crystallized from ethanol or aqueous ethanol. In the case of *chloroacet-N-butylanilide* (25), 2 mol. prop. of the amine were used to 1 mol. prop. of acid chloride, and the product was isolated from the benzene layer after washing with 2·5 N-HCl and water.

Method C. A mixture of the amine (10 g.) and chloroacetic anhydride (1·5–2·0 mol. prop.; Bischoff & Walden, 1894) was heated with conc. H_2SO_4 (1 drop) in an oil bath at 120–140° for 1 hr. While still warm, the reactants were poured into cold water and the precipitated oil triturated with a glass rod. When solidification took place, the resulting solid was collected, washed with water, and crystallized from ethanol or aqueous ethanol. When the crude product remained as an oil, it was isolated by ether extraction and purified by fractional distillation.

Method D. A solution of the chloroacetyl compound (1 mol. prop.) in the minimum of hot ethanol was mixed with a similar solution of KSCN (1·1 mol. prop.), and the mixture was refluxed on a steam bath for 1 hr. Most of the solvent was removed by distillation and the residue cooled and triturated several times in cold water. The pale-yellow plastic residues so obtained often gave considerable trouble in attempts to crystallize them, but purification was generally achieved by obtaining seed crystals from a small sample of the crude material (employing as solvent a mixture of ethanol and ethyl acetate, and using a cooling bath of ethanol: solid CO_2), and then using these seeds for a bulk crystallization with the same solvent and an ice:salt bath.

Method E. The chloroacetyl compound (1 mol. prop.) was added to a solution of NaI (4 mol. prop.) in dry acetone, and the mixture refluxed for 16–20 hr. on a steam bath. The solvent was removed by distillation, the residue triturated thoroughly in cold water, and the crude product finally purified with charcoal and crystallized from ethanol or aqueous ethanol.

Chloroacet-2:4-dichloroanilide (5). Chloroacetanilide (97 g.) and fused sodium acetate (105 g.) were dissolved in glacial acetic acid (750 ml.), and gaseous chlorine was passed in until the solution had absorbed the theoretical quantity for 2 mol. prop. (82 g.). During this process, the temperature of the liquid rose to 60–70°. The reactants were cooled, a stream of SO_2 was passed through the solution to decompose any *N*-chloro compounds, and the mixture poured into a large volume of cold water. The resulting solid was collected, washed with water and crystallized from ethanol (300 ml.); the product was obtained as stellate clusters of fine, colourless needles, m.p. 100° (94 g.; 69% yield). The pure *chloroacet-2:4-dichloroanilide* melted at 102°. (Found: C, 40·2; H, 2·8; N, 6·0. $C_8H_6ONCl_3$ requires C, 40·3; H, 2·5; N, 5·9%.) This compound has also been made by method *A* from 2:4-dichloroaniline. Under the conditions described above, it was not possible to bring about further nuclear chlorination, and additional absorption of chlorine produces an *N*-chlorinated product.

Pentachloroaniline. Conc. HCl (45 ml.) and $SnCl_2$, $2H_2O$ (30 g.) were added to a solution of pentachloronitrobenzene (10 g.) in glacial acetic acid (40 ml.) and the mixture was refluxed vigorously for 10 hr. The reactants were cooled, poured on ice and 5N-NaOH was added until the initial pasty precipitate of tin hydroxides had redissolved. The solid residue was collected, washed with 5N-NaOH, then with water and dried at 100° (8·6 g.; m.p. 226°). Very pale-brown needles were obtained on crystallization from a large volume of ethanol (5·1 g.; 46% yield, m.p. 229°). Willgerodt & Wilcke (1910), who prepared the compound by chlorinating 2:4:6-trichloroaniline, reported a m.p. of 232°. It does not form a picrate.

Chloroacet-2:3:4:5:6-pentachloroanilide (7). Pentachloroaniline (5 g.) was dissolved in dry benzene (45 ml.), finely powdered anhydrous $BaCO_3$ (15 g.) was then added, followed by a solution of chloroacetyl chloride (5 ml.) in dry benzene (10 ml.). The mixture was refluxed for 4 hr., filtered hot, and the solid residue washed with benzene. The combined reaction liquor and washings were washed with water and aqueous $NaHCO_3$, and evaporated to a small volume. The resulting solid, after crystallization from a large volume of ethanol, formed colourless interlacing fine needles (1·6 g.; 25% yield; m.p. 216–217°). The pure *chloroacet-2:3:4:5:6-pentachloroanilide* melted at 217°. (Found: C, 28·4; H, 0·9. $C_8H_3ONCl_6$ requires C, 28·1; H, 0·9%.)

Trichloroacet-N-methylanilide (22). This compound was prepared by method *B* in 45% yield and obtained pure from aqueous ethanol as stellate clusters of colourless prisms, m.p. 71·5–72°. (Found: C, 42·4; H, 3·3. Calc. for $C_9H_8ONCl_3$: C, 42·8; H, 3·2%.) Spiegel & Spiegel (1907) gave m.p. 55°.

Chloroacet-N-pentylanilide (26). *N*-Pentylaniline (30 g.) dissolved in dry benzene (120 ml.) was mixed with a solution of chloroacetyl chloride (7 ml.) in dry benzene (25 ml.). The homogeneous liquid (kept dry with a $CaCl_2$ tube) was refluxed on a steam bath for 16 hr. and allowed to cool. The organic layer was then extracted thoroughly with 2·5N-HCl, washed with water, dried and evaporated. The residual oil, on distillation *in vacuo*, gave two fractions. The main fraction, on redistillation, gave a pale-yellow oil, b.p. 139–141°/1 mm. (13 g.; 59% yield), which proved to be *chloroacet-N-pentylanilide*. (Found: N, 6·1. $C_{13}H_{18}ONCl$ requires N, 5·8%.) The subsidiary fraction, on redistillation, gave an oil, b.p. 117°/1 mm. (2·8 g.) which solidified on standing. Crystallization from a mixture of ethyl acetate and light petroleum (b.p. 80–100°) gave colourless plates, m.p. 124°. (Found: C, 66·1; H, 8·7. $C_{11}H_{17}N.HCl$ requires C, 66·1; H, 9·0%.) The identity of this material as *N-pentylaniline hydrochloride* was confirmed by a mixed m.p. with another sample prepared unambiguously from pentyllaniline in dry ether and HCl.

N-n-*Decylaniline.* A mixture of *n*-decyl bromide (66 g.; 1 mol. prop.) and aniline (138 g.; 5 mol. prop.) was refluxed for 4 hr., cooled, and made alkaline with 5N-NaOH. The organic layer was separated, dried over solid KOH, and fractionally distilled. The pure *N-n-decylaniline* was obtained as a colourless oil, b.p. 150–152°/1 mm. (60 g.; 86% yield). (Found: C, 82·6; H, 11·5. $C_{16}H_{27}N$ requires C, 82·4; H, 11·6%.) The *p-toluenesulphonyl* derivative separated from methanol as colourless monoclinic prisms, m.p. 44·5–46°. (Found: C, 71·6; H, 8·4. $C_{23}H_{33}O_2NS$ requires C, 71·3; H, 8·5%.) The *picrolonate* separated from ethanol as lemon-yellow rhombohedra, m.p. 127–128°. (Found: C, 62·9; H, 6·9. $C_{16}H_{27}N.C_{16}H_8O_9N_4$ requires C, 62·8; H, 7·0%.)

N-Methyl-2:4-dinitroaniline. This improved method of preparation is based on some observations of Valton (1925). Chloro-2:4-dinitrobenzene (10 g.) was dissolved in warm ethanol (100 ml.) and 20% aqueous methylamine added from a burette, while occasionally heating the reaction mixture on a steam bath, until an intense-red colour just persisted after 3 min. heating (about 10 ml. of amine solution were required). The red colour was cleared with a few drops of glacial acetic acid, and the mixture cooled. The product separated as bright-yellow needles (9·2 g.; 95% yield; m.p. 173–174°).

N-Methylpicramide. The following description is more convenient than previous methods (cf. Romburgh, 1883). A solution of picryl chloride (23·3 g.; Boyer, Spencer & Wright, 1946) in warm ethanol (150 ml.) was periodically heated on a steam bath while 20% aqueous methylamine was added slowly from a burette until a deep-red colour just persisted in the solution after boiling for 2 min. The red colour was cleared with a few drops of glacial acetic acid. An oil, which had separated during the reaction, solidified on cooling the mixture. This solid was redissolved by heating and the addition of further ethanol to the reaction liquor. The product separated on cooling as a bright-yellow crystalline mass (21 g.; 93% yield; m.p. 111–112°).

Chloroacet-N-methyl-2:4-dinitroanilide (47). Chloroacet-*N*-methylanilide (18 g.) was added to fuming HNO_3 (100 ml.; sp.gr. 1·5) and the mixture heated on a steam bath under reflux for 30 min., cooled and poured on to ice. A yellow solid separated, which was collected, washed with water and crystallized from methanol. The product weighed 16 g. (60% yield; m.p. 77–79°). The pure *chloroacet-N-methyl-2:4-dinitroanilide* was obtained as bright-yellow prisms which melted at 80°, either alone or on admixture with the product (m.p. 80°), obtained on chloroacetylating 2:4-dinitroaniline by method *C*. This fact establishes the orientation of the two nitro groups entering the nucleus during the nitration. (Found: N, 15·3. $C_9H_8O_5N_3Cl$ requires N, 15·4%.)

Ethyl 4-chloroacetamidobenzoate (73). This compound was prepared by method *A* in 60% yield, but repeated crystal-

lization did not raise the m.p. above 110–111° (Einhorn (1899) reported m.p. 116°). (Found: N, 6·0. Calc. for $C_{11}H_{12}O_3NCl$: N, 5·8%.)

(Phenylthio)acet-N-methylanilide (75). Metallic sodium (2·9 g.) was dissolved in a mixture of thiophenol (13·8 g.) and anhydrous ethanol (25 ml.), and a solution of chloroacet-*N*-methylanilide (23·2 g.) in hot ethanol (100 ml.) added to the resulting solution. After refluxing for 2 hr., the reactants were cooled, poured into cold water and allowed to stand for a few moments, when the precipitated oil solidified. The solid was collected and crystallized from aqueous ethanol. The dry product weighed 27 g. (84% yield; m.p. 71°). A pure sample of *(phenylthio)acet-N-methylanilide* formed colourless triclinic prisms melting at the same temperature. (Found: N, 5·6. $C_{15}H_{15}ONS$ requires N, 5·4%.)

(Phenylthio)acet-N-benzylanilide (76). Metallic sodium (2·1 g.) was dissolved in a mixture of thiophenol (10 g.) and anhydrous ethanol (25 ml.), and a solution of chloroacet-*N*-benzylanilide (23·5 g.) in warm ethanol (100 ml.) added. After refluxing for 2 hr., the mixture was cooled and poured into water. The precipitated oil could not be induced to crystallize, and was therefore isolated by ether extraction and distillation *in vacuo*. The temperature rose rapidly to 240°/1 mm., when a steady fraction was obtained without decomposition. This gave a white crystalline powder (15·0 g.; 50% yield; m.p. 49°). The pure *(phenylthio)acet-N-benzylanilide*, crystallized from a mixture of benzene and light petroleum (b.p. 80–100°) as colourless rhombohedra, had the same m.p. (Found: C, 75·4; H, 5·3. $C_{21}H_{19}ONS$ requires C, 75·7; H, 5·7%.)

Benzenesulphonylacet-N-methylanilide (77). (Phenylthio)acet-*N*-methylanilide (10 g.) was dissolved in glacial acetic acid (50 ml.), aqueous H_2O_2 (11·5 ml., 100 vol.) added slowly during 15 min., the solution allowed to stand for 15 min., and then heated on a steam bath for 2 hr. The cooled solution was poured into water and the precipitated oil scratched to solidify it. The crude product was collected and crystallized from aqueous ethanol, giving colourless monoclinic prisms (7·1 g.; 63% yield; m.p. 120–122°). The pure *benzenesulphonylacet-N-methylanilide* melted at 122–122·5°. (Found: N, 5·0. $C_{15}H_{13}O_3NS$ requires N, 4·8%.)

Benzenesulphonylacet-N-benzylanilide (78). This material was obtained in a manner similar to that described in the preceding paragraph, using (phenylthio)acet-*N*-benzylanilide (5·1 g.) as the starting material. Crystallized from ethanol, the product formed colourless, tetragonal prisms (4·1 g.; 74% yield; m.p. 142°). The pure *benzenesulphonylacet-N-benzylanilide* melted at 144°. (Found: N, 3·9. $C_{21}H_{19}O_3NS$ requires N, 3·8%.)

2:4-*Dichlorophenoxyacet-4-chloroacetamidoanilide* (80). 2:4-Dichlorophenoxyacetyl chloride was obtained essentially by the method of Freed (1946), but the crude residue, after removal of the excess of thionyl chloride, was crystallized from light petroleum (b.p. 60–80°) and not distilled *in vacuo*. By this means, the desired product was obtained as colourless crystals, m.p. 52·5–53·5°. (Found: C, 40·2; H, 2·4. Calc. for $C_8H_5O_2Cl_3$: C, 40·1; H, 2·1%.) Freed (1946) reported m.p. 44·5–45·5°.

2:4-Dichlorophenoxyacetyl chloride (20 g.) was mixed with a suspension of *p*-nitroaniline (25 g.) in dry benzene (450 ml.), and the mixture refluxed on a steam bath for 16 hr. and allowed to cool. The solid was collected, thoroughly shaken, first with 2·5 N-HCl, then aqueous Na_2CO_3, and finally water, dried at 100°, clarified with charcoal, and crystallized from a large volume of benzene. 2:4-*Dichlorophenoxyacet-4-nitroanilide* formed very pale-yellow prisms and plates (20·7 g.; 62% yield; m.p. 200–201°). (Found: C, 49·2; H, 2·8. $C_{14}H_{10}O_4N_2Cl_2$ requires C, 49·3; H, 2·9%.)

The above nitro compound (12·9 g.) was suspended in ethanol (300 ml.) and hydrogenated at ordinary pressure and temperature over Raney nickel. When this reaction was complete, the products were heated until the organic solid dissolved, the catalyst was removed by filtration, and the filtrate was evaporated to dryness. The residue was crystallized from ethanol (8·1 g.; 58% yield; m.p. 138°). Pure 2:4-*dichlorophenoxyacet-4-aminoanilide* formed colourless, interlacing needles, m.p. 140–141°. (Found: C, 54·2; H, 3·9. $C_{14}H_{12}O_2N_2Cl_2$ requires C, 54·0; H, 3·9%.) The *acetyl* derivative separated from ethanol as colourless, hair-like needles, m.p. 217–218°. (Found: C, 54·4; H, 4·0. $C_{16}H_{14}O_3N_2Cl_2$ requires C, 54·6; H, 4·0%.) The *picrate* separated from ethanol as yellow, interlacing needles, which darkened slowly from 170° and melted at 222° (decomp.). (Found: C, 44·7; H, 3·0. $C_{14}H_{12}O_2N_2Cl_2.C_6H_3O_7N_3$ requires C, 44·4; H, 2·8%.)

2:4-Dichlorophenoxyacet-4-aminoanilide (7·1 g.) was dissolved in hot, dry benzene (100 ml.), treated with chloroacetyl chloride (5·4 ml.) and the mixture refluxed for 2 hr. The benzene and excess of chloroacetyl chloride were removed by distillation *in vacuo*, the residue was triturated in water, and the resulting crude solid was crystallized from a large volume of glacial acetic acid.

2:4-*Dichlorophenoxyacet-4-chloroacetamidoanilide* was obtained as buff blades, m.p. 237–238°. (Found: C, 49·4; H, 3·3. $C_{16}H_{12}O_3N_2Cl_2$ requires C, 49·6; H, 3·4%.)

Oximinoacet-N-methylanilide (82). This compound was prepared according to the procedure given by Gilman & Blatt (1941) for the preparation of oximinoacetanilide, and was obtained as colourless prisms, m.p. 169–170°. (Found: C, 60·8; H, 5·6. Calc. for $C_9H_{10}O_2N_2$: C, 60·7; H, 5·6%.) Sandmeyer (1919) recorded m.p. 145°.

Bis(methylphenylcarbamylmethyl) sulphide (83). A hot solution of Na_2S, $9H_2O$ (6·6 g.) in water (10 ml.) was added to a solution of chloroacet-*N*-methylanilide (10 g.) in 50% (v/v) aqueous ethanol (100 ml.), and the mixture refluxed for 1 hr. The ethanol was evaporated and the product recrystallized from ethanol as stellate clusters of colourless prisms, m.p. 112–113° (7·1 g.; 78% yield). A pure sample of *bis(methylphenylcarbamylmethyl) sulphide* melted at 114°. (Found: N, 9·0. $C_{18}H_{20}O_2N_2S$ requires N, 8·6%.)

Bis(benzylphenylcarbamylmethyl) sulphide (84). This compound was prepared in 65% yield using the method described in the preceding paragraph; the starting material in this case was chloroacet-*N*-benzylanilide. The *bis-(benzylphenylcarbamylmethyl) sulphide* separated from ethanol in the form of colourless, monoclinic prisms, m.p. 121–122°. (Found: N, 5·9. $C_{30}H_{28}O_2N_2S$ requires N, 5·9%.)

1:2-*Bis(N-chloroacetylanilino)ethane* (85). A mixture of 1:2-bisanilinoethane (14·7 g.), chloroacetyl chloride (20 ml.) and dry benzene (150 ml.) was refluxed for 2 hr., the solvent removed *in vacuo*, and the residue triturated in water. The resulting solid was collected, washed with water and crystallized from ethanol (19·5 g.; 77% yield; m.p. 152°). The compound, pure 1:2-bis(*N*-chloroacetylanilino)-ethane, formed colourless, hexagonal prisms, m.p. 154–155°. (Found: C, 59·2; H, 4·9. $C_{18}H_{18}O_2N_2Cl_2$ requires C, 59·2; H, 4·9%.)

1:2-*Bis(N-thiocyanoacetylanilino)ethane* (86). 1:2-Bis(*N*-chloroacetylanilino)ethane (13·3 g.) was refluxed for 2 hr. with a solution of KSCN (7·8 g.) in ethanol (200 ml.). The

Table 1. *New compounds*

No.	Name	Method of preparation	Yield (%)	Physical properties	Analyses: Element	Found (%)	Required (%)
6	Chloroacet-2:4:6-trichloroanilide	*B*	72	Colourless interlacing needles, m.p. 180°	C	35·1	35·2
					H	2·05	1·8
					N	5·35	5·1
9	Dichloroacet-2-chloroanilide	*A*	53	Colourless matted needles, m.p. 109–110°	C	40·3	40·3
					H	2·75	2·5
10	Dichloroacet-3-chloroanilide	*A*	60	Colourless prismatic rods, m.p. 100°	C	40·0	40·3
					H	2·2	2·5
					N	6·1	5·9
11	Dichloroacet-4-chloroanilide	*A*	37	Colourless monoclinic prisms, m.p. 136–137°	C	40·3	40·3
					H	2·5	2·5
12	Dichloroacet-2:4-dichloroanilide	*B*	48	Colourless matted needles, m.p. 129°	C	35·2	35·2
					H	1·6	1·8
15	Trichloroacet-2-chloroanilide	*A*	47	Colourless oil, b.p. 136–138°/2·3 mm. Monoclinic prisms, m.p. 61–61·5°	C	35·3	35·2
					H	2·2	1·8
16	Trichloroacet-3-chloroanilide	*A*	47	Colourless glistening rectangular plates, m.p. 100°	C	35·0	35·2
					H	1·9	1·8
17	Trichloroacet-4-chloroanilide	*A*	41	Colourless plates and prisms, m.p. 125–126°	C	35·0	35·2
					H	1·9	1·8
18	Trichloroacet-2:4-dichloroanilide	*B*	43	Colourless monoclinic prisms, m.p. 74–75°	C	31·1	31·2
					H	1·6	1·3
19	Trichloroacet-2:4:6-trichloroanilide	*B*	63	Colourless monoclinic prisms, m.p. 119–120°	C	28·3	28·1
					H	0·9	0·9
24	Chloroacet-*N*-propylanilide	*A*	44	Pale straw-coloured oil, b.p. 160°/12 mm.	N	6·8	6·6
25	Chloroacet-*N*-butylanilide	*B*	40	Pale-yellow oil, b.p. 136–139°/3·5 mm.	N	6·1	6·2
27	Chloroacet-*N*-hexylanilide	*C*	52	Almost colourless oil, b.p. 147°/1·3 mm.	C	66·4	66·3
					H	8·1	7·9
28	Chloroacet-*N*-octylanilide	*C*	30	Almost colourless, oil, b.p. 171–173°/1·9 mm.	C	68·8	68·4
					H	8·6	8·5
29	Chloroacet-*N*-decylanilide	*C*	23	Pale-green oil, b.p. 180–182°/1·7 mm.	C	70·4	69·8
					H	9·3	9·1
30	Chloroacet-*N*-benzylanilide	*A*	65	Colourless plates, m.p. 75–76°	C	69·3	69·4
					H	5·45	5·4
38	Iodoacet-*N*-methylanilide	*E*	70	Colourless rectangular plates, m.p. 57–58°	C	38·9	39·3
					H	3·4	3·6
42	Chloroacet-2:4-dinitroanilide	*C*	77	Pale-yellow needles, m.p. 111–112°	N	16·2	16·2
43	Chloroacet-2:4:6-trinitroanilide	*C*	68	Thin, buff prisms, m.p. 208°	N	18·7	18·4
44	Chloroacet-*N*-methyl-2-nitroanilide	*B*	82	Colourless hexahedra, m.p. 78–79°	C	47·2	47·3
					H	4·0	3·9
45	Chloroacet-*N*-methyl-3-nitroanilide	*B*	81	Pale-cream irregular prisms, m.p. 107–108°	C	47·5	47·3
					H	3·9	3·9
46	Chloroacet-*N*-methyl-4-nitroanilide	*B*	69	Colourless triclinic prisms,	C	47·0	47·3

46	Chloroacet-*N*-methyl-4-nitroanilide	*B*	69	Colourless triclinic prisms, m.p. 109·5–110°	C H	47·0 4·2	47·3 3·9
48	Chloroacet-*N*-methyl-2:4:6-trinitroanilide	*C*	68	Pale-yellow prisms, m.p. 140°	N	17·6	17·6
49	Chloroacet-*N*-ethyl-2:4-dinitroanilide	*C*	42	Lemon-yellow prisms, m.p. 80–81°	C H N	41·8 3·9 14·5	41·8 3·5 14·6
50	Iodoacet-*N*-methyl-2-nitroanilide	*E*	62	Pale-yellow monoclinic prisms, m.p. 72·5–73·5°	C H	33·7 3·0	33·8 2·8
51	Iodoacet-*N*-methyl-3-nitroanilide	*E*	76	Pale-yellow monoclinic prisms, m.p. 91–91·5°	C H	34·1 2·9	33·8 2·8
52	Iodoacet-*N*-methyl-4-nitroanilide	*E*	86	Pale-yellow prisms and plates, m.p. 101–102°	C H	34·0 2·8	33·8 2·8
53	Iodoacet-*N*-methyl-2:4-dinitroanilide	*E*	80	Bright-yellow prisms, m.p. 121–122°	C H N	29·5 2·2 11·4	29·6 2·2 11·5
54	Iodoacet-*N*-methyl-2:4:6-trinitroanilide	*E*	42	Lemon-yellow monoclinic prisms, m.p. 155–156°	C H N	26·9 1·6 13·8	26·3 1·7 13·7
56	Thiocyanoacet-*N*-benzylanilide	*D*	55	Colourless prisms, m.p. 59–60°	N	9·8	9·9
57	Thiocyanoacet-*N*-phenylanilide	*D*	93	Colourless tetragonal prisms, m.p. 87–88°	C H N	67·4 4·4 10·5	67·2 4·5 10·5
58	Thiocyanoacet-*N*-methyl-2-nitroanilide	*D*	62	Irregular buff prisms, m.p. 47·5–48·5°	C H	47·5 3·6	47·8 3·6
59	Thiocyanoacet-*N*-methyl-3-nitroanilide	*D*	87	Irregular colourless plates, m.p. 84·5–85·5°	C H	47·7 3·6	47·8 3·6
60	Thiocyanoacet-*N*-methyl-4-nitroanilide	*D*	71	Irregular colourless prisms, m.p. 113–114°	C H	48·0 3·8	47·8 3·6
61	Thiocyanoacet-*N*-methyl-2:4-dinitroanilide	*D*	54	Pale-yellow prisms, m.p. 95°	N	18·7	18·9
62	Thiocyanoacet-*N*-methyl-2:4:6-trinitroanilide	*D*	64	Pale-yellow prisms, m.p. 131·5°	N	20·0	20·5
63	Thiocyanoacet-*N*-ethyl-2:4-dinitroanilide	*D*	72	Pale-yellow prisms, m.p. 85–85·5°	C H	42·5 3·2	42·6 3·2
66	4-Chloroacetamidodiphenyl	*B*	52	Colourless, pearly plates, m.p. 178–179°	C H	68·1 5·2	68·4 4·9
68	Chloroacet-*N*-methyl-4-hydroxyanilide	*A*	66	Colourless prisms, m.p. 165°	N	7·25	7·0
70	Chloroacet-4-methylthioanilide	*A*	59	Colourless hexagonal blocks, m.p. 134°	N	6·7	6·5
71	Chloroacet-4-thiocyanoanilide	*A*	40	Pale-yellow, rectangular prisms, m.p. 172°	N	12·7	12·4
74	*m*-Chloroacetamido-ααα-trifluorotoluene	*A*	54	Colourless prisms, m.p. 72°	N	6·2	5·9
79	Ethyl 4-chloroacetamidophenoxyacetate	*A*	46	Colourless prisms, m.p. 98·5–99·5°	C H	53·4 5·0	53·0 5·2
90	β-Iodopropionanilide	*E*	90	Colourless rods, m.p. 123–124°	C H	39·5 3·6	39·3 3·6
93	δ-Iodovaleranilide	*E*	52	Colourless needles, m.p. 79–80°	C H	43·1 4·7	43·6 4·6

solvent was removed by distillation, cold water added to the residue, and the resulting solid collected and crystallized from ethanol (13·6 g.; 90% yield; m.p. 163–165°). The pure 1:2-*bis*(N-*thiocyanoacetylanilino*)*ethane* formed long, colourless prisms, m.p. 164–166°. (Found: C, 58·6; H, 4·7. $C_{20}H_{18}O_2N_4S_2$ requires C, 58·5; H, 4·4%.)

δ-*Chlorovaleranilide* (91). A mixture of δ-chlorovaleric acid (154 g.; Gaudry & Berlinguet, 1949) and thionyl chloride (410 ml.) was refluxed on a steam bath for 2 hr., the excess of thionyl chloride distilled at ordinary pressure, and the residue distilled *in vacuo*. δ-*Chlorovaleryl chloride* was obtained as a colourless liquid, b.p. 56–58°/2·5 mm. (144 g.; 83% yield). (Found: C, 38·6; H, 5·2. $C_5H_8OCl_2$ requires C, 38·7; H, 5·2%.)

A solution of aniline (85 ml., 2 mol. prop.) in dry ether (250 ml.) was added slowly to a solution of δ-chlorovaleryl chloride (71 g., 1 mol. prop.) in dry ether (250 ml.) and the mixture was refluxed for 1 hr. The ether was removed by distillation, the residue triturated in water (1 l.) containing conc. HCl (30 ml.), and then collected. The crude solid was crystallized from aqueous ethanol to give a yield of 81 g. (84%; m.p. 105–107°). The pure δ-*chlorovaleranilide* formed colourless, monoclinic prisms, m.p. 107–108°. (Found: C, 62·2; H, 6·8. $C_{11}H_{14}ONCl$ requires C, 62·4; H, 6·8%.)

δ-*Chlorovaler*-N-*methylanilide* (92). This substance was prepared in a manner similar to that described above for δ-chlorovaleranilide, the crude product being isolated by ether extraction. The pure δ-*chlorovaler*-N-*methylanilide* was obtained as a colourless oil, b.p. 149–150°/1·5 mm. (51% yield). (Found: C, 64·3; H, 7·3. $C_{12}H_{16}ONCl$ requires C, 63·9; H, 7·1%.)

Chloromethanesulphon-N-*methylanilide* (94). A solution of chloromethanesulphonyl chloride (20 g.; Johnson & Douglass, 1941) and methylaniline (27·2 g.) in dry benzene (200 ml.) was refluxed on a steam bath for 6 hr. The solvent was then evaporated, and the residual oil twice fractionally distilled. The pure *chloromethanesulphon*-N-*methylanilide* was thus obtained as a pale-yellow oil, b.p. 125–127°/1·0 mm. (10·8 g.; 37% yield). (Found: C, 43·5; H, 4·7; N, 6·7. $C_8H_{10}O_2NClS$ requires C, 43·8; H, 4·6; N, 6·4%.)

Chloromethanesulphon-N-*ethylanilide* (95). This compound was obtained as a pale-yellow oil, b.p. 130°/0·9 mm., by employing a method similar to that described in the preceding paragraph (38% yield). (Found: C, 46·7; H, 5·4; N, 6·1. *Chloromethanesulphon*-N-*ethylanilide*, $C_9H_{12}O_2NClS$, requires C, 46·3; H, 5·1; N, 6·0%.)

Biological methods

The methods described below were regarded as routine screening methods only and, whilst quantitative, the results obtained were used as a guide for further research.

The two methods were adopted to show the effects of the chemicals upon two different morphological features of fungi, viz. the mycelium and the spores. Only in the case of one test fungus were the tests carried out on both mycelium and spores. The fungi for the mycelial test were selected because of their regular, well-shaped colonies in culture and broad representation of pathogenic types. The organisms used for the spore germination test were chosen for their abundant spore production and general ease of management.

Formulation of materials. When the compound was a solid or a non-volatile liquid it was taken up in waterproofed limestone powder to give a dust containing 10% active principle. In cases where that percentage was impossible lower ones had to be made. The dust base itself (containing about 1% lanoline and supplied by The Derbyshire Stone Co. Ltd., Matlock) has been found to be inactive, and it was assumed that the chemical under test underwent no change in form or activity. This is not to imply that formulation for practical use plays no part in activity, but for the laboratory tests, only the minimum amount of formulation was carried out to facilitate application of the compounds to the test organism. This dust formulation was used for one of the tests, while for the other the chemical was used in a pure state, as a solution in a suitable solvent, e.g. acetone, ethanol, $CHCl_3$, or water.

Mycelial test. This was based on a technique described by Forsberg (1949). In this method, pieces of sterilized button thread were plated out on malt-agar plates and these were inoculated with small pieces of the test fungus. These plates were then incubated at 23° for as long as was necessary for the surface of the agar to be covered with the fungal growth. During this period the mycelium probably grew in between the fibres of cotton in the thread and certainly over the outside, the outcome being that the pieces of thread were transformed into relatively manageable pieces of test organism. These lengths of thread were removed from the plates with sterile forceps and cut into pieces about $\frac{1}{2}$ in. long with sterilized scissors.

These small pieces were rolled in the dust containing the chemical under test in such a manner that the piece was completely covered with dust, the excess removed by tapping, and the pieces replated, five to each malt-agar plate. The pieces were so arranged that growth could take place from each piece, yielding reasonably large circular colonies. The incubation period, which varied from fungus to fungus, was governed by the time taken for the individual colonies to reach a size which just avoided intermingling of mycelium at the periphery of the colonies (see Pl. 4). At this stage measurements of colony diameters, in two directions at right angles, were made on all pieces, numbering ten per treatment, and the totals so obtained were compared one with the other.

Untreated controls were included in every set of plates, and a known fungicide was periodically included as a check on the technique. Two chemicals have been found satisfactory over the range of fungi, these being ferric dimethyldithiocarbamate (Fermate) and tetramethylthiuram disulphide (TMTD). These likewise were used as 10% dusts in the same base as the test chemicals.

In the assessment of the results of this test it was obvious that a chemical which allowed no growth in the test plates possessed fungicidal activity, whilst growth of the same order as the controls suggested complete lack of activity. The intermediate stages were arrived at by estimating the amount of inhibition, i.e. the difference between treated and untreated size, as a percentage of the untreated figure. The formula

$$\frac{C-T}{C} \times 100,$$

where C = untreated values, and T = treated values gave an estimate of the degree of control exerted by the chemical under test.

For a series of chemicals, therefore, a range of percentage inhibition was obtained and numbered according to the following schedule: 1 = 98–100%, 2 = 93–97%, 3 = 51–92%, 4 = 0–50%.

Fig. 1. Forsberg technique. Colony growths of test fungus (*Fusarium nivale*) from infected pieces of thread, showing the stage at which diameter measurements are taken.

Table 2 shows that a broad outline of the activity of any compound is easily obtained. This was sufficient for the purpose of studying the correlation between structure and activity.

The fungi used in this test were as follows: *Pythium debaryanum* Hesse, *Corticium solani* (Prill & Delacr.)Bourd. & Galz. =*Rhizoctonia solani* Kühn, *Botrytis cinerea* Fr., *Fusarium nivale* Auct., *F. caeruleum* (Lib.) Sacc., *Helminthosporium avenae* Eidam. Of these, the first-named is the most rapid growing and 2–3 days incubation sufficed, whilst the slower ones, *Fusarium nivale*, and *Helminthosporium* needed 7 or so days before the colonies reached an adequate size.

Spore germination test. This method was based on that laid down by the American Phytopathological Society (1943) and included some of the techniques used by Miller (1949) and Evans & Martin (1935). The method ensured the intimate association of test organism and chemical without the inclusion of any filler or inert base. It is more accurate and is capable of recording activity at a very low concentration of test chemical, but required more preparation than the first method.

The fundamental requirement of this method was absolute cleanliness of all the apparatus used. All the glassware used in this technique was cleaned out with chromic/nitric acid cleaning fluid, thoroughly washed with running water, rinsed several times with distilled water and finally washed with water, twice distilled in Pyrex glass. The test tubes and slides used were respectively filled with, and immersed in, doubly distilled water and autoclaved at 20 lb./sq.in. for 30 min. and then oven-dried. (At one time heating in 5% (w/v) KOH solution was included in the cleaning schedule, but it was discovered that the highly polished surface of the slides deteriorated fairly rapidly, giving rise to scratches and imperfections in which traces of chemicals from previous trials could remain and thus prejudice any further results. This KOH wash was therefore omitted.) The slides were then allowed to stand in pure $CHCl_3$ for a few minutes to remove traces of grease, removed and air-dried, dipped in ethanol and flamed, and placed under cover to await use. It was found that rigid adherence to this cleaning procedure was essential for consistent results.

The above method was used where the glass surface itself was the substrate upon which the test solutions were placed, but in certain cases, and also to control the spreading of liquid drops, it was necessary to coat the slides with a cellulose film. This was achieved by dipping the slides in a 2·5% (w/v) solution of cellulose nitrate in butyl acetate (Evans & Martin, 1935) and drying quickly in dry heat. When rapid drying was not carried out, the film adsorbed water vapour from the air and rapidly became opaque, thus impeding microscopic examination. These slides were again kept under cover until used.

The production of spores for this test followed the American recommendations in so far that the fungi were cultured on potato-dextrose agar slopes and incubated for 7 days at a temperature of 22°. These conditions appeared to be the optimum values for spore production (McCallan & Wilcoxon, 1939). The spores were removed from the slope by adding distilled water and rubbing the culture surface with a glass rod. This suspension was strained through muslin to remove fragments of mycelium and agar and the resulting suspension was centrifuged at 2000 rev./min. for about 5 min. The liquid was decanted and replaced with fresh distilled water, the spores resuspended and recentrifuged. This process was repeated three or four times in order to ensure that all traces of nutrient solution from the agar medium were washed from the spores thereby avoiding the possibility of differential germinative capacity resulting from varying amounts of stimulant material included in the suspension. As germination was rather uncertain in distilled water, a synthetic stimulant was added in all cases to the spores for the actual test. This consisted of a solution containing 0·001% (w/v) potassium or sodium citrate, and 0·1% (w/v) sucrose (Miller, 1949). As this was the final dilution required a stock solution 100 times the recommended strength was made, autoclaved, and then stored in a refrigerator at about 3° and diluted as required for use.

It was shown that the actual density of the spore suspension influenced the germinating capacity of the organism and that a final concentration of 50000 spores/ml. was a satisfactory value (McCallan & Wilcoxon, 1939). In all tests, the suspension was adjusted to this figure by dilution from the original suspension calculated from counts made on a haemocytometer cell.

As pure chemicals only were involved in this test, the first procedure was to make a solution in a suitable solvent. For this particular series acetone was used throughout and a stock solution of chemical prepared. The usual test strengths were 50, 25 and 15 p.p.m., but in some cases where the strongest solution failed to reveal activity differences relative to chemical structure, tests were carried out using 200 p.p.m. solution. Thus, stock solutions of 200 p.p.m. chemicals were made up and used, as such, or diluted to give 50, 25 and 15 p.p.m. where necessary.

For these tests the cleaned, uncoated glass slides were used and on to each of the three slides used for each chemical, 0·25 ml. was pipetted. The low surface tension of acetone ensured that the liquid spread over the whole surface, and as the slides were levelled beforehand, the chemical was assumed to be distributed evenly. The acetone was allowed to evaporate, thus giving a deposit of chemical of 0·6–0·7 μg./sq.cm. when a 50 p.p.m. solution was used. (The fungicidal standard in this test was copper, usually as the sulphate; it had been found (Byrde, 1952) that 0·2 μg./sq.cm. was sufficient to prevent 50% of the spores germinating, and from previous experience it was obvious that 0·6 μg./sq.cm. of copper would completely inhibit germination. It could thus be assumed that test chemicals showing high activity at the various concentrations compared well with copper under the conditions of test.) The spore suspensions, of correct density and made up in stimulant solutions of requisite concentration, were pipetted on to the treated surfaces, three drops to a slide, each drop being 0·05 ml. and spreading to about 1 cm. in diameter. The slides were then distributed at random on glass racks, placed in the incubation chamber which maintained a high relative humidity, the chamber was sealed with a vaselined lid and the whole incubated at 22° for a period of 17–24 hr. (The period of incubation does affect the germination to some extent but as large differences in activity were sought, the effect of an incubation period of even 48 hr. was relatively unimportant.)

After the incubation period the spores were examined microscopically using a $\frac{2}{3}$ in. objective and $\times 10$ eyepiece. The total number of spores in each field was counted, sufficient fields being examined to give a total of 150 per slide (i.e. about 450/treatment) and the spores divided into germinated and ungerminated groups. In all tests there was an untreated set of slides, to show the germination capacity of the spores themselves. If this figure was not in the region of 95–100%, the test was regarded as invalid.

In order to compare successive tests, the percentage of germinated spores was calculated for each treatment and the percentage of spores inhibited from germination was also calculated by expressing the difference between untreated and treated germination percentages as a fraction of the untreated percentage, thus

$$\frac{C-T}{C} \times 100,$$

where C = untreated germination percentage, and T = treated germination percentage. This had the effect of removing from the final figures the effect of the few spores in each batch which invariably failed to germinate and produced a result based on the assumption that all the spores were viable and that failure to germinate was occasioned by the treatment alone.

This procedure helped to minimize the effect of using different lots of spores on different days. The same categories were adopted as for the mycelial test.

Only one fungus found a use in both tests. This was *Botrytis cinerea* whilst the other two used in the test were *Sclerotinia fructigena* Aderh. & Ruhl. and *Alternaria brassicae* (Berk.) Sacc. All three fungi were easily cultured but the latter tended to be spasmodic in spore production and sometimes cultures consisted solely of white vegetative mycelium. *S. fructigena* proved to be most sensitive to fungicidal activity.

RESULTS

The antifungal activities of the ninety-five organic compounds tested are given in Table 2 (mycelial test) and Table 3 (spore germination test). The effects of nuclear chlorination on chloroacetanilide (1), which may be regarded as a reference compound, can be seen in the activities of compounds 2–7 (Table 2). The excellent control effected by compound 1 on all the six experimental fungi was maintained on introducing a chlorine atom either ortho or meta to the chloroacetamido group, but this activity was diminished with the substituent in the para position, as was the case when two chlorine atoms were present in the nucleus (5). Further halogenation completely destroyed the activity (6, 7). The same general tendency was observed with the dichloroacetanilides (8–13) and the trichloroacetanilides (14–19), though in these cases the demarcation was not so regular. This may be due to the fact that these two groups of compounds were considerably less active than the chloroacetanilides. Polychlorination in the acetyl group reduced the overall activity of these compounds, the maximum activity being exhibited by the monochloroacetyl derivatives (acetanilide itself is inactive). This effect is particularly well illustrated by the *N*-methylanilides (20–22).

The activities of a series of *N*-substituted homologues of chloroacetanilide have been determined (Table 3; 20, 23–29) as biological activity can increase considerably in such a series. In this particular instance, the general control of the fungi was poor at the concentration of 200 p.p.m., but a definite maximum of control of *S. fructigena* was obtained with chloroacet-*N*-pentylanilide (26). This series was not continued beyond chloroacet-*N*-decylanilide. Two other compounds of the same type (30, 31, Table 2) also showed little activity.

The effect of different halogen atoms in the nucleus is illustrated with compounds 32, 4, 33 and 34 (Table 2), containing one such atom in the *para* position of the nucleus. The fluorine compound (32) had the same activity as the chlorine compound (4), but the substitution of a bromine atom (33) reduced this activity considerably, while it disappeared completely in the iodine compound (34).

A more important change in biological activity was shown on changing the halogen atom in the acetyl group. Here, the mycelial test (Table 2) demonstrated that the high activity of chloroacetanilide (1) is maintained in bromoacetanilide (35) and iodoacetanilide (36). Further, the activity of the last two compounds was only slightly diminished when their concentration was reduced to 50 p.p.m. in the spore germination test (Table 3), while the activity of chloroacetanilide (1) had disappeared. The same effects were noted with the *N*-methyl derivatives (20, 37, 38).

The effects of nuclear nitration upon fungicidal activity were well illustrated by compounds 1, 39–43 (Table 2), where a marked similarity with the effects of nuclear chlorination was noticeable. Nitro groups in the *ortho* and *meta* positions of the rings did not reduce the activity of the parent compound (1), but when the substituent was *para* to the chloroacetamido moiety, the activity was slightly reduced (41), while the introduction of further nitro groups ultimately destroyed the activity completely (42, 43). The corresponding group of *N*-methyl derivatives (20, 44–48) were submitted to the spore germination test. Here, the much lower concentration employed (200 p.p.m.) masked the activity of even the potentially active members of the series, which was uniformly inactive. However, a similar series of nitro derivatives (50–54) based on iodoacet-*N*-methylanilide (38), which had already been seen to be more active than chloroacet-*N*-methylanilide (20), did manifest considerable activity at 200 p.p.m. (Table 3). Here, again, increasing nuclear substitution tended to reduce the activity exhibited by the mono-substituted compounds.

The effects of various other nuclear substituents upon the activity of chloroacetanilide has been examined with compounds 67–73 (Table 2), and 74, 79, 80 (Table 3). None of these derivatives showed an enhanced activity, while most of them were much weaker fungicides. Replacement of the benzene nucleus by naphthalene (64, 65) and diphenyl (66) likewise resulted in no improved activity. The union of two chloroacetanilide molecules into one molecule by an ethylene bridge (85) also produced no marked activity.

Table 2. *Results of mycelial test*

(Materials applied as dusts containing 10% chemical in an inert base.)

1 = 98–100% Inhibition A = Good general activity
2 = 93–97% Inhibition B = Fair general activity
3 = 51–92% Inhibition C = Some activity
4 = 0–50% Inhibition D = Little or no activity

No.	Name	*Fythium debaryanum*	*Corticium solani*	*Botrytis cinerea*	*Fusarium nivale*	*Fusarium caeruleum*	*Helmintho-sporium avenae*	Overall symbol
1	Chloroacetanilide	1	2	1	1	1	2	A
2	Chloroacet-2-chloroanilide	1	2	2	2	1	2	A
3	Chloroacet-3-chloroanilide	1	2	1	1	1	1	A
4	Chloroacet-4-chloroanilide	3	4	2	3	1	1	B
5	Chloroacet-2:4-dichloroanilide	3	3	1	2	2	3	B
6	Chloroacet-2:4:6-trichloroanilide	4	4	4	4	4	4	D
7	Chloroacet-2:3:4:5:6-pentachloroanilide	4	4	4	4	4	4	D
8	Dichloroacetanilide	1	1	3	4	4	1	C
9	Dichloroacet-2-chloroanilide	4	3	4	4	4	3	D
10	Dichloroacet-3-chloroanilide	1	1	3	1	3	1	B
11	Dichloroacet-4-chloroanilide	3	2	3	3	4	3	C
12	Dichloroacet-2:4-dichloroanilide	4	3	4	4	4	3	D
13	Dichloroacet-2:4:6-trichloroanilide	4	4	4	4	4	3	D
14	Trichloroacetanilide	1	2	3	1	4	1	B
15	Trichloroacet-2-chloroanilide	3	1	3	4	4	3	C
16	Trichloroacet-3-chloroanilide	3	2	4	3	4	2	C
17	Trichloroacet-4-chloroanilide	3	3	4	4	4	3	D
18	Trichloroacet-2:4-dichloroanilide	3	1	4	4	4	3	D
19	Trichloroacet-2:4:6-trichloroanilide	3	1	3	3	4	1	C
20	Chloroacet-*N*-methylanilide	1	1	1	1	1	1	A
21	Dichloroacet-*N*-methylanilide	2	2	3	4	4	3	C
22	Trichloroacet-*N*-methylanilide	3	2	3	4	4	3	D
30	Chloroacet-*N*-benzylanilide	4	3	4	4	4	4	D
31	Chloroacet-*N*-phenylanilide	3	2	4	4	3	4	D
32	Chloroacet-4-fluoroanilide	.	1	4	3	4	1	B
33	Chloroacet-4-bromoanilide	.	2	4	4	4	4	C
34	Chloroacet-4-iodoanilide	.	4	4	4	4	4	D
35	Bromoacetanilide	.	1	1	1	1	1	A
36	Iodoacetanilide	.	1	1	1	1	1	A
37	Bromoacet-*N*-methylanilide	.	1	1	1	1	1	A
38	Iodoacet-*N*-methylanilide	.	1	1	1	1	1	A
39	Chloroacet-2-nitroanilide	1	1	3	1	1	2	A
40	Chloroacet-3-nitroanilide	1	1	3	1	1	1	A
41	Chloroacet-4-nitroanilide	1	1	4	3	3	1	B
42	Chloroacet-2:4-dinitroanilide	.	3	3	3	3	1	C
43	Chloroacet-2:4:6-trinitroanilide	.	4	4	4	4	3	D
55	Thiocyanoacet-*N*-methylanilide	1	1	2	3	2	2	A
56	Thiocyanoacet-*N*-benzylanilide	3	4	4	4	4	4	D
67	Chloroacet-4-hydroxyanilide	.	2	4	4	4	1	C
68	Chloroacet-4-hydroxy-*N*-methylanilide	.	4	4	4	4	3	D
69	Chloroacet-4-methoxyanilide	3	2	3	3	4	1	C
70	Chloroacet-4-methylthioanilide	4	1	4	3	4	3	C
71	Chloroacet-4-thiocyanoanilide	3	3	4	3	4	3	C
72	4-Chloroacetamido-*NN*-dimethylaniline	3	1	4	4	4	2	C
73	Ethyl 4-chloroacetamidobenzoate	.	1	4	3	3	1	B
83	Bis(methylphenylcarbamylmethyl) sulphide	.	4	4	4	4	4	D
84	Bis(benzylphenylcarbamylmethyl) sulphide	4	4	4	4	4	4	D

Table 3. *Results of spore germination test*

1 = 98–100% Inhibition
2 = 93–97% Inhibition
3 = 51–92% Inhibition
4 = 0–50% Inhibition

A = Good general activity
B = Fair general activity
C = Some activity
D = Little or no activity

BC = *Botrytis cinerea*
SF = *Sclerotinia fructigena*
AB = *Alternaria brassicae*

No.	Name	Concentration of chemical (p.p.m.) 200 BC	200 SF	200 AB	50 BC	50 SF	50 AB	25 BC	25 SF	25 AB	15 BC	15 SF	15 AB	Overall symbol
1	Chloroacetanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
20	Chloroacet-*N*-methylanilide	4	3	4	4	3	4	3	3	4	4	4	4	D
23	Chloroacet-*N*-ethylanilide	4	4	4	4	4	4	.	.	.	.	.	.	D
24	Chloroacet-*N*-propylanilide	4	4	4	4	4	4	.	.	.	.	.	.	D
25	Chloroacet-*N*-butylanilide	4	3	4	4	4	4	.	.	.	.	.	.	D
26	Chloroacet-*N*-pentylanilide	4	2	4	4	4	4	.	.	.	.	.	.	D
27	Chloroacet-*N*-hexylanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
28	Chloroacet-*N*-octylanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
29	Chloroacet-*N*-decylanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
32	Chloroacet-4-fluoroanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
33	Chloroacet-4-bromoanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
34	Chloroacet-4-iodoanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
35	Bromoacetanilide	.	.	.	1	1	3	1	1	3	2	1	4	B
36	Iodoacetanilide	.	.	.	1	1	3	1	1	3	2	1	4	B
37	Bromoacet-*N*-methylanilide	.	.	.	1	2	4	4	3	4	4	4	4	C
38	Iodoacet-*N*-methylanilide	.	.	.	1	1	4	4	3	4	4	3	4	C
43	Chloroacet-2:4:6-trinitroanilide	.	.	.	4	4	3	4	4	4	.	.	.	D
44	Chloroacet-*N*-methyl-2-nitroanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
45	Chloroacet-*N*-methyl-3-nitroanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
46	Chloroacet-*N*-methyl-4-nitroanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
47	Chloroacet-*N*-methyl-2:4-dinitroanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
48	Chloroacet-*N*-methyl-2:4:6-trinitroanilide	.	.	.	4	3	4	4	4	4	.	.	.	D
49	Chloroacet-*N*-ethyl-2:4-dinitroanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
50	Iodoacet-*N*-methyl-2-nitroanilide	1	1	3	.	.	.	.	.	.	.	.	.	B
51	Iodoacet-*N*-methyl-3-nitroanilide	1	1	1	.	.	.	.	.	.	.	.	.	A

52	Iodoacet-*N*-methyl-4-nitroanilide	1	1	1	.	.	.	.	.	.	.	.	.	A
53	Iodoacet-*N*-methyl-2:4-dinitroanilide	1	1	4	.	.	.	.	.	.	.	.	.	B
54	Iodoacet-*N*-methyl-2:4:6-trinitroanilide	1	4	4	.	.	.	.	.	.	.	.	.	C
55	Thiocyanoacet-*N*-methylanilide	4	3	4	.	.	.	.	.	.	.	.	.	D
57	Thiocyanoacet-*N*-phenylanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
58	Thiocyanoacet-*N*-methyl-2-nitroanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
59	Thiocyanoacet-*N*-methyl-3-nitroanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
60	Thiocyanoacet-*N*-methyl-4-nitroanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
61	Thiocyanoacet-*N*-methyl-2:4-dinitroanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
62	Thiocyanoacet-*N*-methyl-2:4:6-trinitroanilide	.	.	.	4	4	4	4	4	4	.	.	.	D
63	Thiocyanoacet-*N*-ethyl-2:4-dinitroanilide	4	4	4	.	.	.	.	.	.	.	.	.	D
64	Chloroacet-α-naphthylamide	.	.	.	4	4	4	.	.	.	.	.	.	D
65	Chloroacet-β-naphthylamide	.	.	.	4	4	4	.	.	.	.	.	.	D
66	4-Chloroacetamidodiphenyl	.	.	.	4	4	4	.	.	.	.	.	.	D
74	*m*-Chloroacetamido-ααα-trifluorotoluene	.	.	.	4	4	4	.	.	.	.	.	.	D
75	(Phenylthio)acet-*N*-methylanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
76	(Phenylthio)acet-*N*-benzylanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
77	Benzenesulphonacet-*N*-methylanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
78	Benzenesulphonacet-*N*-benzylanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
79	Ethyl 4-chloroacetamidophenoxyacetate	.	.	.	4	4	4	.	.	.	.	.	.	D
80	2:4-Dichlorophenoxyacet-4-chloroacetamidoanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
81	Oximinoacetanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
82	Oximinoacet-*N*-methylanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
85	1:2-Bis(*N*-chloroacetylanilino)ethane	.	.	.	4	4	4	.	.	.	.	.	.	D
86	1:2-Bis(*N*-thiocyanoacetylanilino)ethane	.	.	.	4	4	4	.	.	.	.	.	.	D
87	β-Chloropropionanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
88	β-Chloropropion-*N*-methylanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
89	β-Bromopropionanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
90	β-Iodopropionanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
91	δ-Chlorovaleranilide	.	.	.	4	4	4	.	.	.	.	.	.	D
92	δ-Chlorovaler-*N*-methylanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
93	δ-Iodovaleranilide	.	.	.	4	4	4	.	.	.	.	.	.	D
94	Chloromethanesulphon-*N*-methylanilide	.	.	.	4	4	4	.	.	.	.	.	.	D
95	Chloromethanesulphon-*N*-ethylanilide	.	.	.	4	4	4	.	.	.	.	.	.	D

Changes have been made in the monovalent substituent in the acetyl group. The most interesting of these was the replacement of the halogen atom by the thiocyano group. This change did not, apparently, affect the fungicidal activity associated with the parent chloroacet-*N*-methylanilide (20), for thiocyanoacet-*N*-methylanilide (55) exhibited good general activity in the mycelial test (Table 2), and only poor activity at 200 p.p.m. in the spore germination test (Table 3). No enhancement of activity has been detected with other thiocyanoacetyl compounds, namely 56 (Table 2), 57 and 86 (Table 3), and the series of nitro derivatives 58–63 (Table 3). A number of other changes in the acetyl substituent did not produce any interesting increases of activity (75–78, 81, 82, Table 3; 83, 84, Table 2).

A small group of compounds (87–93) was prepared for the purpose of observing the change of fungicidal properties on moving the halogen atom in the halogenoacetanilides away from the carbonyl group. Table 3 shows that the considerable activity exhibited by bromoacetanilide (35) completely vanished in β-bromopropionanilide (89), and the same was equally true for the analogous iodo compounds (36, 90); nor did the activity reappear on moving the iodine atom even further away from the carbonyl group, as in δ-iodovaleranilide (93).

Two compounds (94, 95) were prepared in which the carbonyl group of chloroacetanilide had been replaced by the sulphonyl group. This change was accompanied by no increase in fungicidal properties.

DISCUSSION

The biological activities recorded in Table 2 confirm the claims of Maier-Bode (1940) that, in general, the halogenoacetanilides do possess fungicidal activity when formulated as 10 % dusts in inert solid diluents. This activity, however, was only maintained in a few cases when the concentration of the active constituent was reduced to 200 p.p.m. or smaller values. It has also been confirmed that chloroacetanilide is less active than chloroacet-*N*-methylanilide, but *N*-alkylation did not always result in increased activity, e.g. trichloroacetanilide was more active than trichloroacet-*N*-methylanilide.

Leonard & Blackford (1949) have shown that chloroacetanilide inhibited the growth of *Aspergillus niger* more efficiently than did dichloroacetanilide which, in turn, was more efficient than trichloroacetanilide. These findings have been confirmed for other fungi in the present work, which has demonstrated that nuclear chlorination also reduces the activity. In addition, it has confirmed the superior activity of bromoacetanilide, which was equalled by that of iodoacetanilide.

The molecular size of the test chemical naturally affects its biological activity since, if the active halogenoacetamido group becomes masked by a large nucleus, the compound will be inactive. Thus, no significant activity was found in compounds with a high molecular weight. The effect of increasing the molecular weight by substitution is well illustrated in two series of compounds, where the continued introduction of chlorine atoms (1–7) and nitro groups (39–43) gradually reduced a significant activity to zero.

Most of the chemicals described in this work have undesirable physiological properties. It was expected that the halogenoacetyl derivatives would be inherent lachrymators and skin irritants, but they occasioned no marked unpleasantness during synthesis because of their low volatility. In the form of dusts and sprays, however, the skin irritancy became very noticeable, a fact which would have precluded their practical application. (The same type of problem has been encountered by McCallan, 1950.) When the halogen atom in the acyl radical is not adjacent to the carbonyl group, as in β-iodopropionanilide, the compound did not exhibit irritancy, but unfortunately it was also no longer a fungicide.

The unpleasant physiological properties seem to be greatly diminished or eliminated completely in the nitro derivatives of the halogenoacetanilides, where appreciable fungicidal activity was maintained by the mononitro compounds. Skin irritation was also removed by replacing the halogen atom in the acetyl radical by the thiocyano group. The resulting thiocyanoacet-*N*-methylanilide had the same order of activity as chloroacet-*N*-methylanilide but, like the latter, this activity faded on dilution to 200 p.p.m.

SUMMARY

1. The fungicidal activities of ninety-five non-metallic organic compounds derived structurally from acetanilide have been tested; sixty-two of these compounds are new, and their preparation and physical characteristics are described.

2. Two biological methods have been employed: (*a*) the mycelial test, in which the chemical was used as a 10 % dust in an inert solid diluent, and (*b*) the spore germination test, in which the chemical was used as an acetone solution at concentrations of 200 p.p.m. or less.

3. This group of compounds exhibits general fungicidal activity at the higher concentration of 10 %, but few of the chemicals retain their activity at 200 p.p.m.

4. Most of the compounds, particularly in the form of dusts and sprays, possess unpleasant physiological properties which precludes any practical application.

The authors wish to thank Miss A. P. Wilson and Dr H. A. Stevenson for their encouragement and interest in the work, and Miss J. Keefe and Mr B. E. Leggetter for technical assistance.

REFERENCES

American Phytopathological Society (1943). *Phytopathology*, **33**, 627.
Bischoff, C. A. & Walden, P. (1894). *Ber. dtsch. chem. Ges.* **27**, 2949.
Boyer, R., Spencer, E. Y. & Wright, G. F. (1946). *Canad. J. Res.* **24** B, 200.
Byrde, R. W. J. (1952). *J. hort. Sci.* **27**, 130.
Einhorn, A. (1899). German Patent, 106502.
Evans, A. C. & Martin, H. (1935). *J. Pomol.* **13–14**, 261.
Forsberg, J. L. (1949). *Phytopathology*, **39**, 172.
Freed, V. H. (1946). *J. Amer. chem. Soc.* **68**, 2112.
Gaudry, R. & Berlinguet, L. (1949). *Canad. J. Res.* **27** B, 285.
Gilman, H. & Blatt, A. H. (1941). *Organic Syntheses*, collective vol. I, 2nd ed., pp. 327–329. London: Chapman and Hall.
Jacobs, W. A. & Heidelberger, M. (1917). *J. Amer. chem. Soc.* **39**, 1439.
Johnson, T. B. & Douglass, I. B. (1941). *J. Amer. chem. Soc.* **63**, 1571.
Leonard, J. M. & Blackford, V. L. (1949). *J. Bact.* **57**, 339.
McCallan, S. E. A. (1950). *Contr. Boyce Thompson Inst.* **16**, 5.
McCallan, S. E. A. & Wilcoxon, F. (1939). *Contr. Boyce Thompson Inst.* **11**, 5.
Maier-Bode, H. (1940). German Patent, 695907.
Miller, H. J. (1949). *Phytopathology*, **39**, 245.
Romburgh, P. v. (1883). *Rec. Trav. chim. Pays-Bas*, **2**, 105.
Sandmeyer, T. (1919). *Helv. chim. Acta*, **2**, 239.
Spiegel, L. & Spiegel, P. (1907). *Ber. dtsch. chem. Ges.* **40**, 1734.
Swensen, A. D. & Weaver, W. E. (1948). *J. Amer. chem. Soc.* **70**, 4060.
Valton, P. A. (1925). *J. chem. Soc.* **127**, 41.
Weaver, W. E. & Whaley, W. M. (1947*a*). *J. Amer. chem. Soc.* **69**, 515.
Weaver, W. E. & Whaley, W. M. (1947*b*). *J. Amer. chem. Soc.* **69**, 1144.
Willgerodt, C. & Wilcke, K. (1910). *Ber. dtsch. chem. Ges.* **43**, 2754.

The Deamidation of Nicotinamide by Bacteria

By D. E. HUGHES and D. H. WILLIAMSON

Medical Research Council Unit for Research in Cell Metabolism, The University, Sheffield

(*Received* 21 *May* 1953)

In a previous paper from this laboratory it was shown that nicotinamide was rapidly broken down to nicotinic acid by washed suspensions of *Lactobacillus arabinosus* 17–5 (Hughes & Williamson, 1952*a*). In the present paper it is shown that the breakdown of nicotinamide in this micro-organism is hydrolytic, nicotinic acid and ammonia being formed stoicheiometrically. Cell-free extracts which deamidate nicotinamide have been prepared and some properties of the enzyme investigated. A number of other species of lactobacilli were tested and all found to deamidate nicotinamide. In contrast to this, none of eleven other species of bacteria, with the single exception of a strain of *Staphylococcus albus*, were active.

EXPERIMENTAL

Organisms and growth media. *Lactobacillus arabinosus* 17–5, *Lb. bulgaricus*, National Collection of Type Cultures, strain 76, *Lb. delbrueckii*, NCTC, strain 5125, *Lb. casei*, NCTC, strain 8001, and *Lb. helveticus*, NCTC, strain 8103, two locally isolated strains of *Staphylococcus aureus* and NCTC, strain 6775, several locally isolated strains of *Staph. albus* and haemolytic streptococci were grown at 37° on an enzymic casein hydrolysate medium (Nossal, 1951) adjusted to pH 7·6. In some experiments *Lb. arabinosus* was grown on the semi-synthetic medium used for the microbiological assay of nicotinic acid (McIlwain & Hughes, 1948). Twelve strains of lactobacilli isolated from beer by Dr T. K. Walker, Manchester College of Technology, were grown at 30° on the casein hydrolysate medium adjusted to pH 5·0. *Acetobacter aceti*, *Aceto. acetosum*, *Aceto. capsulatum*, *Aceto. suboxydans* and *Aceto. turbidens* (supplied by Dr T. K. Walker) were grown at 30° on the yeast extract-glucose medium described by Walker & Tosic (1945). *Proteus morgani*, NCTC, strain 2812, and a local strain of *P. vulgaris* and *Escherichia coli* were grown on a medium consisting of acid-hydrolysed casein (McIlwain & Hughes, 1944) and 2% yeast autolysate. A mutant strain of *Aerobacter aerogenes*, exacting for nicotinic acid, was grown on the inorganic salt medium described by Devi & Higginbottom (1949). Three locally isolated strains of *Pseudomonas fluorescens* were grown on the medium of Koser & Baird (1944).

The organisms were harvested by centrifugation, washed twice and suspended in 0·9% (w/v) NaCl to give a suspension containing from 5 to 40 mg. dry wt./ml.

Estimation of enzyme activity. The rate of deamidation of nicotinamide was estimated by measuring ammonia formation: 0·01 M nicotinamide (0·5 ml.), and 0·2 M phosphate buffer, pH 7·0 (1·0 ml.) were placed in one side of a double side-arm test tube (Hughes & Williamson, 1952*b*) and the enzyme solution (0·5 ml. previously adjusted to pH 7) in the other. After 5 min. incubation in a water bath at 40° the contents of the side arms were mixed. The reaction was stopped after varying periods of incubation by the addition of 1·0 ml. of 2 N-H_2SO_4 and ice-cooling. The ammonia was distilled in a modified Parnas apparatus, the distillate nesslerized, and the colours read in a Beckman spectrophotometer at 625 mμ. Ammonia was also estimated in the

reagents and enzyme material. Formation of ammonia was found to proceed linearly when the enzyme concentration was such as to decompose from 10 to 80% of the added 5 μmoles of nicotinamide in 60 min. (Fig. 1). The rate of reaction was proportional to enzyme concentration under these conditions (Fig. 2). In experiments with organisms other than the lactobacilli and where the rate of reaction did not exceed 0·5 μmoles NH_3/mg. dry wt. cells/hr.; 5 times the amount of the above reagents and up to 20 mg. dry wt. cells were incubated up to 2 hr. The results throughout the paper are expressed as μmoles NH_3/mg. dry wt. cells/hr.; where extracts were used results are expressed in terms of mg. dry wt. of the cells from which the extract was made.

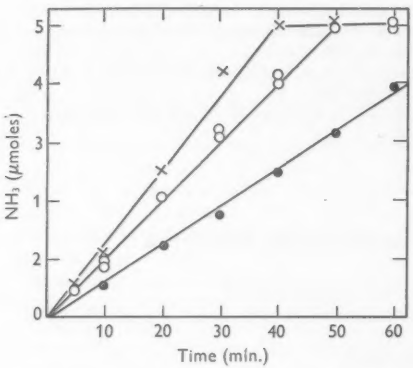


Fig. 1. Formation of ammonia from nicotinamide by various enzyme preparations. Each tube contained 0·5 ml. 0·01 M nicotinamide, 0·5 ml. 0·8 M phosphate buffer, pH 7·0, and 1·0 ml. enzyme preparation as indicated. ○—○, intact cells; ●—●, acetone powder; ×—×, cell-free extract.

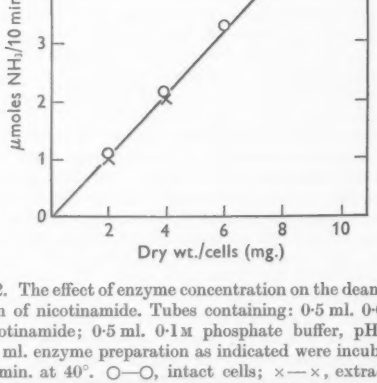


Fig. 2. The effect of enzyme concentration on the deamination of nicotinamide. Tubes containing: 0·5 ml. 0·01 M-nicotinamide; 0·5 ml. 0·1 M phosphate buffer, pH 7·0; 1·0 ml. enzyme preparation as indicated were incubated 10 min. at 40°. ○—○, intact cells; ×—×, extract of cells prepared by grinding with powdered glass.

Chemicals. Nicotinic acid and nicotinamide were the purified specimens used in previous work (Hughes & Williamson, 1952*a*). Diphosphopyridine nucleotide (DPN) (50% pure) was found to be contaminated with traces of acetone which interfered with the ammonia estimations. To remove acetone the DPN was ground in ice-cold ethanol and washed with ice-cold ether in a centrifuge tube, then dried for 3 days over P_2O_5 in a vacuum desiccator. All other chemicals were commercial specimens and were not purified further.

Preparation of cell-free extracts. In preliminary experiments, extracts were prepared from cells of *Lb. arabinosus* broken by grinding with powdered glass or by crushing at −20° in the bacterial press (Hughes, 1951). In later experiments it was found more convenient to prepare the extracts from cells dried from the partially frozen state over P_2O_5 (Hughes & Williamson, 1952*b*). The final method for extraction was as follows: dried cells (500 mg.) were ground in a glass pestle and mortar with 6–8 ml. of 0·2 M phosphate buffer, pH 6·0. The volume was made up to 40 ml. with phosphate buffer; 6 drops of toluene were added and the suspension was incubated at 37° in a stoppered flask for 22 hr. It was then centrifuged and the supernatant was decanted. The residue was washed with 10 ml. water on the centrifuge. The combined supernatants contained 80% of the original enzyme activity. They were cooled to 0° to −2° and stirred with 5 vol. ice-cold redistilled acetone. After standing for 10 min. the precipitate was collected by centrifugation, washed with ice-cold acetone and dried in a desiccator over P_2O_5. The dried powder weighed 40–50 mg. and contained 50–60% of the original enzymic activity. About 10% of the enzymic activity was lost from the dried acetone powder and from the dried cells after storage for 10 months at 2°. For subsequent use the dried powder (50 mg.) was triturated in cold distilled water (2·0 ml.), left at 2° for 1 hr. and then centrifuged to remove a little insoluble material and the clear supernatant diluted to 5 ml.

Estimation of nicotinic acid in the presence of nicotinamide. As shown by Larson & Haag (1944) many pyridine compounds when treated with CNBr form yellow or orange derivatives with typical absorption spectra in the region of 400–450 mμ. It was found that this yellow derivative from nicotinic acid treated with NaOH formed material absorbing strongly in the near ultraviolet. It is probably material which acts as an internal filter and quenches the fluorescence of nicotinamide treated under similar conditions (Chaudhuri & Kodicek, 1949). The absorption spectrum of the reaction produced from nicotinic acid has a peak at 372 mμ. (ϵ_{372}, where ϵ is molar extinction coefficient $= 3{\cdot}02 \times 10^4$), that of nicotinamide is weakly absorbing in this region and decreases and becomes negligible at 400 mμ. (Hughes & Williamson, 1952*a*). By choosing suitable wavelengths, nicotinic acid and nicotinamide may be separately estimated and nicotinic acid may be estimated in a solution containing nicotinamide.

Preparation of the CNBr *reagent.* The reagent as generally prepared (Chaudhuri & Kodicek, 1949) gave erratic results mainly owing to differences in pH between batches and to changes in pH during the reaction. Consistent and maximum values were obtained when the pH of the reagent was buffered strongly at pH 5·2–5·8. The most suitable buffer was a mixture of 0·05 M acetate and phosphate; succinate and citrate buffers inhibited the reaction. To prepare the CNBr solution, 25 ml. of bromine water were cooled to 2° and 2 M-NaCN was added dropwise until the colour just dis-

appeared. A solution containing 12·5 ml. of 0·5 M phosphate buffer, pH 6 and 12·5 ml. of 0·2 M acetate buffer, pH 5 was then added and the pH finally adjusted to 5·5 (glass electrode) with either NaOH or 10% acetic acid. The reagent when stored in a dark bottle at 2° remained stable for 4–5 days.

Reaction with CNBr. The optimum concentration of CNBr reagent was found to be higher than that generally used in the Koenig reaction. Consistent values were found when 1·0 ml. of the test solution containing from 0·3 to 400 μmoles of nicotinic acid were allowed to react with 1·0 ml. of the buffered reagent. The optimum temperature for the reaction was 70°. Under these conditions the ϵ_{372} increased rapidly up to 15 min., reached a maximum value in 18 to 20 min. and decreased again if heating was continued longer than 25 min.

Reaction with NaOH. Upon addition of the optimum concentration of 6 N-NaOH (0·5 ml.) after reaction with CNBr solutions became yellow, the yellow colour then faded and ϵ_{372} increased and reached 90% of the maximum value in 45 min. Thereafter the rate of increase was slow and the maximum value was reached in 2·5–3 hr.; the ϵ_{372} then decreased and after 16–20 hr. at room temperature had fallen to 80% of the maximum value. With more NaOH the reaction was faster but did not reach the maximum value; with less NaOH the reaction was slow and again did not reach maximum value.

Method. To 0·2–1·0 ml. of the test solution containing from 0·3 to 400 mμmoles of nicotinic acid in a stoppered test tube were added 1·0 ml. of the CNBr reagent and water to make the volume to 2·0 ml. The mixture was heated 20 min. at 70°, cooled in running water; 0·5 ml. of 6 N-NaOH was added and the final vol. was made to 3·0 ml. with water. The tubes were then placed in the dark at room temperature for 45 min. and the optical density was measured in a Beckman spectrophotometer.

The nicotinic acid content of solutions was estimated by comparing the ϵ_{372} with nicotinic acid standards set up at the same time. Solutions containing from 0·3 to 400 mμmoles nicotinic acid obeyed Beer's Law (Fig. 3) and duplicate determinations agreed to $\pm$0·5%. Results for solutions containing both nicotinic acid and nicotinamide did not agree with the expected values when calculated by the use of Morton & Stubbs (1946) equations. This is probably owing to the fluorescence of nicotinamide and the quenching effect of nicotinic acid (Chaudhuri & Kodicek, 1949). In the present work, however, advantage was taken of the fact that during the enzymic reaction, it was the proportion of acid to amide that changed whilst the substituted pyridine content of the solutions remained constant. Standard solutions containing varying proportions of nicotinamide and nicotinic acid were treated with CNBr and NaOH and their extinctions measured at wavelengths between 350 and 400 mμ. An approximately straight line was given at 400 mμ. (Fig. 4). The nicotinic acid content of solutions from the enzymic deamidation of nicotinamide was therefore estimated from such a standard curve set up at the same time. Duplicate estimations agreed within 2% and the method thus compared favourably with the more tedious microbiological method previously used (McIlwain & Hughes, 1948; Hughes & Williamson, 1952*a*).

Interfering substances. When treated with CNBr and NaOH as described, nicotinamide riboside, nicotinamide ribotide and DPN had absorption spectra like that of nicotinamide. The spectra of nicotinyl hydrazide, nicotinuric acid and 5-fluoronicotinic acid were identical with that

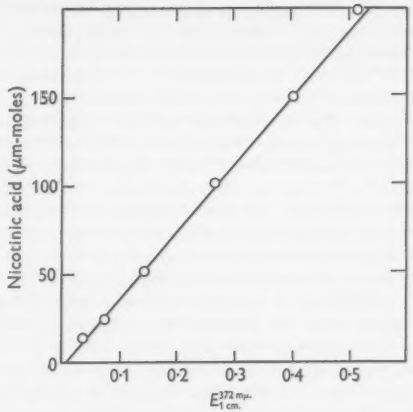


Fig. 3. Standard curve for estimating nicotinic acid prepared by reacting 0·5 ml. of solution with CNBr + NaOH. Final vol. 3·5 ml. $E_{1\,cm.}$ was measured at 372 mμ. in a Beckman or Unicam spectrophotometer against a reagent blank.

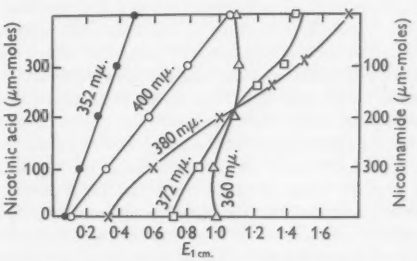


Fig. 4. Standard curves of a mixture of nicotinic acid and nicotinamide reacted with CNBr + NaOH and read at various wavelengths.

of nicotinic acid. Nicotinamide N^1-methochloride, trigonelline, pyridoxine, pyridoxal, 2-fluoronicotinic acid and 6-fluoronicotinic acid did not react with CNBr and NaOH to give material adsorbing in the regions studied. Thiamine reacted to give an absorption spectrum with an ill-defined peak in the region of 370 mμ. Solutions of various salts and casein hydrolysate did not interfere, but solutions containing glucose which had been autoclaved at 120° adsorbed too strongly for nicotinic acid to be estimated.

RESULTS

The deamidation of nicotinamide by various micro-organisms

Deamidation by lactobacilli. Under the standard conditions already described, *Lb. arabinosus* and *Lb. bulgaricus* formed ammonia at rates of 1–2 μmoles/mg. dry wt./hr. (Table 3). All the other lactobacilli tested formed ammonia, but at slower

rates (0·1–0·8 μmole/mg. dry wt./hr.). Because *Lb. arabinosus* was consistently the most active the following experiments were done with this organism.

To test the stoicheiometry of the reaction, ammonia and nicotinic acid were measured at time intervals during reaction between nicotinamide and acetone powder of an extract of *Lb. arabinosus*; both products were formed stoicheiometrically (Table 1). Similar results were obtained with washed cells and other cell-free extracts. In addition, nicotinic acid was measured in some of the tests with the other lactobacilli and was always found to be formed to the same extent as the ammonia.

The identity of nicotinic acid was confirmed by isolation from the reaction fluid, 50 ml. of 0·01 M nicotinamide in 0·05 M phosphate buffer, pH 7·0, being incubated at 37° with 50 mg. of acetone powder preparation until ammonia estimation showed the reaction to be complete (16 hr.). The mixture was made acid to pH 4·0 with N hydrochloric acid, evaporated to dryness *in vacuo* at 40° and dried by evaporating it to dryness twice with acetone. The dried material was then extracted with three successive portions of boiling acetone. The combined extracts were filtered and taken to dryness in a test tube. The bottom of the test tube was immersed in an oil bath at 210° and the white solid which sublimed was collected, re-crystallized from water twice and dried *in vacuo* over phosphorus pentoxide: m.p. 198°; mixed m.p. 198°. The absorption spectra of solutions of the crystals in N hydrochloric acid and of solutions in N sodium hydroxide were identical with similarly prepared nicotinic acid. Tests by the paper-chromatographic methods of Kodicek & Reddi (1951) also supported the evidence that the crystals were those of nicotinic acid. The deamidation of nicotinamide is thus a hydrolytic reaction in which ammonia and nicotinic acid are formed. Under the conditions of the test nicotinamide is decomposed completely. There was no inhibition of the rate of reaction in the presence of high concentrations of either nicotinic acid or ammonia (0·1 M). Likewise no formation of nicotinamide could be detected when the various enzyme preparations were incubated with ammonium nicotinate either alone or with adenosine triphosphate, glutamine and salts of magnesium and manganese.

The effect of growth conditions on the deamidation of nicotinamide. No significant difference was found in the rate of ammonia formation from nicotinamide by *Lb. arabinosus* when the cells were grown on the semi-synthetic medium or the casein-hydrolysate medium to which had been added nicotinic acid, nicotinamide or DPN in concentration from 1×10^{-3} to 5×10^{-7} M. The ability of the cells to deamidate nicotinamide does not therefore depend on the concentration of nicotinic acid or its derivatives in the medium as was found to be the case with DPN synthesis (Hughes & Williamson, 1952*a*). Nor did the age of the cells (5–24 hr., Table 2), the absence of glucose or pH of the media, have any marked effect. Similar results were obtained with *Lb. bulgaricus*. Nicotinamide deamidase thus may be considered to be a 'constitutive' enzyme of lactobacilli.

Table 1. *Nicotinic acid and* NH_3 *formation from nicotinamide*

(Each double-arm tube contained 0·5 mg. acetone powder from *Lb. arabinosus*, 8·0 ml. 0·2 M phosphate buffer pH 7·0 in one side arm and 2 ml. 0·01 M nicotinamide in the other arm. The contents were mixed after 5 min. at 40° and the reaction was stopped by the addition of 2 ml. 5 N-H_2SO_4 at the times indicated. Nicotinic acid was determined in a 1 ml. sample by reaction with CNBr; NH_3 was determined in 8·0 ml.)

Time after mixing (min.)	NH_3 formed (μmoles/10 ml.)	Nicotinic acid formed (μmoles/10 ml.)
0	1·1	1·2
15	2·1	2·3
45	3·8	4·3
90	7·8	8·2
150	11·8	11·8
180	13·2	13·8

Table 2. *The effect of age of culture on the rate of deamidation by* Lactobacillus arabinosus 17–5

(Hydrolysed casein medium (1 l.) was inoculated with 1 ml. of a 24-hr. culture and shaken at 37°. Samples were taken for the turbidimetric estimation of growth at 0·5 hr. periods and larger samples for the estimation of enzyme activity were taken as indicated. The cells were collected and washed and their enzyme activity was estimated as described.)

Time (hr.)	Growth (mg. dry wt. bacteria/ml. medium)	Rate of deamidation (μmoles NH_3/mg. dry wt./hr.)
5	0·25	1·3
8	0·40	1·1
10	0·45	1·4
12·5	0·8	1·8
14·5	0·9	1·3
24	1·0	1·3

Deamidation of nicotinamide by micro-organisms other than lactobacilli. As shown in Table 3 none of the other organisms tested deamidated nicotinamide at a significant rate (0·05 μmole/mg. dry wt./hr.) with the single exception of one strain of *Staph. albus*. It is noteworthy that some of the organisms tested and found not to deamidate nicotinamide (e.g. staphylococci and streptococci), decompose DPN with the formation of nicotinamide as one of the end products (McIlwain & Hughes, 1948). In contrast to this, *Lb. arabinosus* does not decompose DPN but is able to deamidate nicotinamide at a rapid rate. It seems possible that the nicotinamide deamidase is concerned in the regulation of the

DPN content of the cells (see Hughes & Williamson, 1952*a*). The inability of the three strains of *Ps. fluorescens* to deamidate nicotinamide explains why these organisms do not oxidize nicotinamide when they are adapted to oxidize nicotinic acid. Attempts to isolate from the soil organisms which would grow upon nicotinamide as a sole source of carbon and nitrogen were not successful (cf. Koser & Baird, 1944; Jakoby, Schatz, Hutner & Weber, 1952.)

Table 3. *Deamidation of nicotinamide by various bacteria*

(Organisms were grown for 16 to 24 hr. on the media described in the text, collected, washed and suspended in saline to give 5–20 mg. dry wt./ml. Enzyme activity was measured as described in the text. Maximum and minimum rate of at least three different batches are given.)

Organism	Rate of deamidation (μmoles NH_3/mg. dry wt./hr.)
Lb. arabinosus	2·0–3·0
Lb. casei	0·5–1·0
Lb. delbrueckii	0·5–1·5
Lb. helveticus	0·5–1·0
Lb. bulgaricus	0·5–1·0
Lactobacillus sp. (from yogurt)	1·0–2·0
Lactobacillus strains W1, W2, K	0·3
Lactobacillus strains D4, W6, E4	0·5–1·0
Lactobacillus A4	0·4
Lactobacillus 15	0·1
Staph. albus sp.	0·8–1·5
Staph. albus (7 strains)	<0·05
Staph. aureus (5 strains)	<0·05
Strep. haemolyticus (2 strains)	<0·05
Cl. welchii	<0·05
Esch. coli	<0·05
Aceto. suboxydans, Aceto. capsulatum, Aceto. aceti	<0·05
Aerobacter aerogenes	<0·05
Ps. fluorescens	<0·05

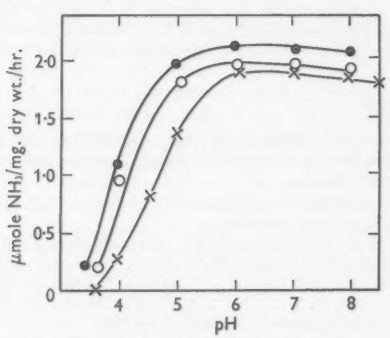


Fig. 5. pH-activity curve of various enzyme preparations. Each tube contained: 0·5 ml. 0·01 M nicotinamide; 0·5 ml. 0·8 M acetate buffer (from pH 3·5 to 5·0) or 0·5 ml. 0·8 M phosphate buffer (from pH 5·0 to 8·0); 1·0 ml. enzyme preparation as indicated. Incubated 10 min. at 40°. ○—○, intact cells; ●—●, acetone powder; ×—×, cell-free extract.

Properties of the enzyme

The pH-*activity curve.* The deamidation reaction in intact cells, in extracts of cells prepared by grinding them with powdered glass, and in extracts of the acetone-dried cells were identical and had a broad optimum between pH 8·0 and 4·5. Below pH 4·5 the activity of the enzyme fell abruptly (Fig. 5).

Substrate concentration curves. The initial rates of reaction at substrate concentrations between 0·025 M and 0·0005 M in intact cells and cell-free extracts obeyed the Michaelis-Menten equation, and the apparent Michaelis constant was 0·0015 M.

Specificity. No ammonia was produced when the acetone-dried cells or the extracts prepared from them were incubated with the following substances: glutamine, asparagine, arginine, adenine, urea, nicotinuric acid, nicotinamide methochloride, trigonelline, nicotinamide riboside, DPN, pyridine-3-sulphonamide, 5-fluoronicotinamide. Because the enzyme did not deamidate the nicotinamide moiety of DPN and nicotinamide riboside, some experiments were made to test the usefulness of the enzyme for determining free nicotinamide in extracts of tissues. Recovery of added nicotinamide (0·1–10 μmoles) from aqueous extracts of various rat tissues was satisfactory but the ammonia blanks were high. The experiments were discontinued since the method appeared to be less convenient than chemical estimations on fractions separated chromatographically (Kodicek & Reddi, 1951; Wollish, Schmall & Shafer, 1951).

Stability. Aqueous suspensions of the freshly washed cells lost about 15–25 % of their original enzyme activity in 7 days when stored at 2°. The cell-free preparation was stable under these conditions. All enzyme preparations lost their activity completely in 3 min. at 100°; about 50 % of activity was lost after 15 min. at 60°.

Solutions of the enzyme did not lose activity when dialysed for 2 days at 2° against tap or distilled water, 0·05 M phosphate buffer, pH 7·0, or 0·05 M acetate buffer, pH 5·0. Treating the enzyme solutions with ethanol, ammonium sulphide or acid and alkaline solutions of ammonium sulphate resulted in irreversible loss of activity. There was no evidence of any cofactor in the preparations.

The effect of inhibitors. The most effective inhibitors of deamidation in intact cells and cell-free extracts of *Lb. arabinosus* were ions of heavy metals, copper sulphate, mercuric chloride and silver nitrate, which inhibited deamidation completely in concentrations between 1×10^{-3} and 5×10^{-4} M. Substances related to nicotinamide such as pyridine-3-sulphonic acid, pyridine-3-sulphonamide, nicotinyl hydrazide, 5-fluoronicotinic acid, 2-fluoronicotinic acid, 6-fluoronicotinic acid did not inhibit in concen-

trations up to 5×10^{-2}M. The phenolphthaleins and sulphonophthaleins which inhibit the glutaminase of *Clostridium welchii* (Hughes & Williamson, 1952*b*) and the acridines, euflavine and atebrine which inhibit renal glutaminase (Archibald, 1944) did not affect deamidation of nicotinamide in concentrations up to 4×10^{-2}M. Sodium azide, hydrazine sulphate, sodium nitrate, sodium iodoacetate, cetyltrimethylammonium bromide, and sodium dodecyl sulphate were also without effect.

DISCUSSION

Although nicotinamide can give rise to nicotinic acid in the intact animal (Ellinger & Abdel-Kader, 1949; Johnson & Lin, 1951; Leifer, Roth, Hogness & Corson, 1951) no enzyme hydrolysing the amide has as yet been described. The present experiments are the first demonstration of a nicotinamide deamidase. It is remarkable that the distribution of the enzyme in bacteria appears to be restricted mainly to the genus *Lactobacillus* where the rate of deamidation can be such that the bacteria decompose their own weight in nicotinamide in 1 to 2 hr. It seems likely that part at least of the nicotinic acid formed from nicotinamide in the intact animal may be formed by the action of lactobacilli present in the digestive tract. It has recently been claimed that nicotinamide is deamidated *in vitro* by rat liver (Quagliariello, Guerritore & Porcellati, 1952). The activity of liver in this respect cannot however be high because nicotinamide is stable enough in the liver to be isolated as an end product of the breakdown of cozymase (Mann & Quastel, 1941) and to serve as a methyl acceptor in experiments on methylation (Ellinger & Armitage, 1953).

The method for the determination of nicotinic acid described in the present paper was elaborated before the chromatographic methods (Kodicek & Reddi, 1951; Wollish *et al.* 1951) were known. The latter methods are more convenient in many ways and of wider application. But there is still scope in the present method as an independent one for identifying and estimating metabolic products of pyridine derivatives (Chaudhuri & Kodicek, 1949; Hughes, 1951).

SUMMARY

1. Washed suspensions and cell-free extracts of *Lactobacillus arabinosus* 17–5 contain an enzyme which hydrolyses nicotinamide to ammonia and nicotinic acid.

2. The enzyme does not attack other naturally occurring nicotinamide derivatives such as diphosphopyridine nucleotide, nicotinamide riboside and nicotinamide ribotide.

3. The pH optimum of the enzyme is broad and between pH 5 and 8. The apparent Michaelis constant is 0·0015M.

4. The formation of nicotinamide deamidase in *Lb. arabinosus* and *Lb. bulgaricus* is not affected by the composition of the growth medium; the enzyme is constitutive.

5. Seven species of lactobacilli tested formed the enzyme. With the exception of a strain of *Staphylococcus albus*, strains of eleven species of other bacteria did not possess the enzyme.

6. A modified Koenig reaction was used to estimate the quantity of nicotinic acid in the presence of nicotinamide. The use of the method for identifying and estimating pyridine derivatives is briefly discussed.

The authors wish to thank Prof. H. A. Krebs, F.R.S., for his interest and suggestions during the course of this work and to acknowledge the technical assistance of Mr M. Clegg. Thanks are also due to Dr T. K. Walker (Manchester) for his gifts of some of the organisms used in this work.

REFERENCES

Archibald, R. M. (1944). *J. biol. Chem.* **154**, 657.
Chaudhuri, P. K. & Kodicek, E. (1949). *Biochem. J.* **44**, 343.
Devi, P. & Higginbottom, C. (1949). *Biochem. J.* **45**, 391.
Ellinger, P. & Abdel-Kader, M. M. (1949). *Biochem. J.* **44**, 627.
Ellinger, P. & Armitage, P. (1953). *Biochem. J.* **53**, 588.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Hughes, D. E. & Williamson, D. H. (1952*a*). *Biochem. J.* **51**, 330.
Hughes, D. E. & Williamson, D. H. (1952*b*). *Biochem. J.* **51**, 45.
Jakoby, W. B., Schatz, A., Hutner, S. H. & Weber, M. M. (1952). *J. gen. Microbiol.* **6**, 278.
Johnson, B. C. & Lin, P. H. (1951). *Fed. Proc.* **10**, 203.
Kodicek, E. & Reddi, K. K. (1951). *Nature, Lond.*, **168**, 475.
Koser, S. A. & Baird, G. R. (1944). *J. infect. Dis.* **75**, 250.
Larsen, P. S. & Haag, H. B. (1944). *Indust. Engng Chem. (Anal.)* **16**, 86.
Leifer, E., Roth, L. J., Hogness, D. S. & Corson, M. H. (1951). *J. biol. Chem.* **190**, 595.
McIlwain, H. & Hughes, D. E. (1944). *Biochem. J.* **38**, 187.
McIlwain, H. & Hughes, D. E. (1948). *Biochem. J.* **43**, 60.
Mann, P. J. G. & Quastel, J. H. (1941). *Biochem. J.* **35**, 502.
Morton, R. A. & Stubbs, A. L. (1946). *Analyst*, **71**, 348.
Nossal, P. M. (1951). *Biochem. J.* **50**, 349.
Quagliariello, G., Guerritore, D. & Porcellati, G. (1952). *2nd Int. Congr. Biochem. Abstr.* p. 227.
Walker, T. K. & Tosic, J. (1945). *J. Inst. Brew.* **51**, 245.
Wollish, E. G., Schmall, M. & Shafer, E. G. E. (1951). *Analyt. Chem.* **23**, 768.

The Phosphorylation and Absorption of Sugars in the Rat

1. HEXOKINASE ACTIVITY IN THE INTESTINAL MUCOSA

By M. P. HELE*
Biochemical Laboratory, University of Cambridge

(*Received* 14 *January* 1953)

Glucose and galactose are known to be more rapidly absorbed from the small intestine of the rat than fructose, mannose and the pentoses (e.g. Cori, 1925). The more rapid absorption of the first two sugars has been attributed to their undergoing phosphorylation in the intestinal mucosa during absorption, a process named 'active' or 'selective' absorption. The less rapidly absorbed sugars have been considered to pass through the mucosa by a process of diffusion, known as 'passive' or 'non-selective' absorption (Verzár & McDougall, 1936). The evidence produced for the direct participation of sugar phosphorylation in selective absorption consists mainly of studies upon the effect of iodoacetate poisoning (Wilbrandt & Laszt, 1933), or phlorrhizin poisoning (Lundsgaard, 1933; Wertheimer, 1933) on sugar absorption *in vivo*, and upon the accumulation of phosphorylated sugars in the mucosa during sugar absorption (Laszt & Sullmann, 1935; Lundsgaard, 1939; Beck, 1942). Whilst indicating that the phosphorylation of sugar in the mucosa is probably a necessary concomitant of sugar absorption, this evidence is not of itself sufficient to demonstrate that phosphorylation participates directly in sugar absorption as part of the transfer mechanism. Other investigators have shown that the distinction once drawn between active and passive absorption may not be so clear as was once thought. Esters are known to accumulate in the mucosa during the absorption of fructose (Lundsgaard, 1939; Kjerulf-Jensen, 1942) and other passively absorbed sugars (Verzár & Sullman, 1937). Xylose, in the cat, can be as rapidly absorbed as glucose (Davidson & Garry, 1940).

If the absorption of sugars from the small intestine involves the direct phosphorylation of these sugars, then it might be expected that the phosphorylation rates of different sugars *in vitro* would bear the same relationship to each other, as do their absorption rates *in vivo*. The demonstration of such a relationship has been attempted here. The results reported fall into two groups, the first dealing with the many factors concerned in the development of an assay for hexokinase activity in dispersions of intestinal mucosa, the second with a more detailed study of the hexokinase. The terms 'phosphatase' and 'ATPase' are used to designate certain activities present in such dispersions, not as identification for specific enzymes.

* Benn W. Levy Student.

EXPERIMENTAL

Piebald Norwegian rats were used, of both sexes, and weighing 130–200 g. They were maintained on the following synthetic diet: 40% rice starch, 17% sucrose, 23% light white casein, 15% arachis oil, 5% salt mixture, 7·5% dried yeast, 2·5% cod-liver oil. A 150 g. rat consumed about 15 g. of this mixture (dry weight) daily. The animals were not fasted before the experiments.

Preparation of tissue suspensions. The animal was killed by decapitation, and the whole length of the small intestine excised and placed in cold, glass-distilled water. The intestine was opened and gently cleaned with a paintbrush. The intestine was blotted with filter paper, weighed to the nearest 0·5 g., returned to clean water, and cut into short lengths. The pieces were ground for 3 min. by hand with an equal weight of water in a glass homogenizer kept on ice. The piston had a smooth surface and fitted loosely. The preparation was squeezed through muslin to remove the pieces of muscle layer. About 8–16 ml. were obtained, containing about 250 mg. wet tissue/ml. The hexokinase remained active towards glucose for 2 hr., whilst the ATPase and phosphatase were stable for at least 2 days. Bacteriological examination showed that contamination with *Escherichia coli* and *Streptococcus faecalis* was negligible. Histological examination showed that after 3 min. treatment in the homogenizer the columnar epithelium alone was split from the mucosa, the cores of the villi remaining behind, attached to the muscle layer, which appeared to be intact. Very few intact cells were observed in the suspension. All of the suspensions used were prepared from single animals.

Assay of hexokinase activity. The final procedure for the determination of hexokinase activity was as follows. Tissue suspensions were incubated in test tubes at 30° in the following mixture: 0·05 M veronal-acetate buffer, pH 8·0, 0·01 M-$MgCl_2$, 0·06 M-NaF, sugars of varying concentrations (0·0014–0·028 M) and adenosine triphosphate (ATP) of varying concentration (0·013–0·033 M); the final volume was 2 ml., containing 0·7 ml. tissue suspension, equivalent to about 175 mg. wet tissue; fluoride was added last so as to avoid the formation of a precipitate (Potter, 1947). Samples of 0·5 ml. were removed at intervals and treated with $Ba(OH)_2$ and $ZnSO_4$ to remove protein and phosphorylated sugar before the estimation of free sugar by the method of Nelson (1944). The rate of the hexokinase reaction was followed by the rate of disappearance of free sugar. It was not found practicable to follow the reaction by the dis-

appearance of ATP because of the high concentration of ATP which had to be used in order to counteract the effect of ATPase.

For the study of the reaction with galactose, suspensions were prepared with the magnesium-Ringer solution described by Meyerhof & Geliazkowa (1947). Bicarbonate was omitted, as suspensions prepared with bicarbonate-containing Ringer were viscous and inactive. Suspensions prepared with water did not phosphorylate galactose, but those prepared with magnesium-Ringer did so, although the rate fell off after the first 2·5 min. All determinations of the phosphorylation rate of galactose were made for this time interval, and most of the suspensions so examined were also tested with glucose and fructose under the same conditions. The rates observed with these two latter sugars with magnesium-Ringer suspensions did not differ from the rates found with water suspensions.

Assay of phosphatase and ATPase activity. A test system similar to that employed for the determination of the hexokinase activity was used, without NaF, and with the appropriate ester or ATP added instead of sugar and ATP. For the phosphatase reaction 0·5 ml. samples were treated with twice the volumes of $Ba(OH)_2$ and $ZnSO_4, 7H_2O$ prescribed by Nelson (1944) so as to ensure the removal of all the phosphorylated sugar and the free sugars were then determined by Nelson's method. For the ATPase reaction the samples were deproteinized with 5% trichloroacetic acid, and the inorganic and 9 min.-labile P estimated by the method of Fiske & Subbarow (1925). The rates of these reactions were expressed as 'mg. free sugar (or inorganic phosphate) formed/ml. tissue suspension/unit of time'.

Preparation of ATP. ATP was prepared from rabbit muscle according to Bailey (1949). The ATP so obtained was contaminated with a metal, probably iron, which seriously interfered with the hexokinase reaction. This metal was removed by stirring the ATP with 8-hydroxyquinoline in $CHCl_3$ (Waring & Werkman, 1942). The ATP was finally precipitated as the sodium salt, using a mixture of equal volumes of ether and ethanol, 9 vol. of mixture to 1 vol. of ATP solution.

Sugars. The sugars used, D-glucose, D-galactose, D-fructose, D-mannose, and D-xylose, were Kerfoot's 'bacteriological sugars'.

Factors affecting the determination of hexokinase activity in the intestinal mucosa

Blanks and controls. Four tissue suspensions, examined by the Nelson procedure, gave no appreciable reducing value when fresh, or after incubation with veronal-acetate buffer or with inorganic phosphate, 0·033 M and 0·016 M. These phosphate concentrations represent the limits of inorganic phosphate appearing in the digest in the presence of ATP during the hexokinase reaction. One fresh suspension contained 0·09 mg. inorganic P/ml. which rose to 0·12 mg./ml. after 10 min. incubation. Small amounts of 9 min.-labile P and 3 hr.-hydrolysable P were found, amounting to 0·01–0·02 mg./ml. suspension.

Incubation of glucose or fructose (0·0055 M) with ten different suspensions in the absence of ATP did not lead to any disappearance of sugar, although in the presence of ATP rapid sugar disappearance was observed. Incubation of 0·028 M glucose (four suspensions) or 0·028 M fructose (two suspensions) with 0·65 M disodium phosphate gave no disappearance of free sugar, although good sugar disappearance was found with ATP, and the presence of an active phosphatase was indicated by the rapid hydrolysis of added glucose 6-phosphate or fructose 6-phosphate.

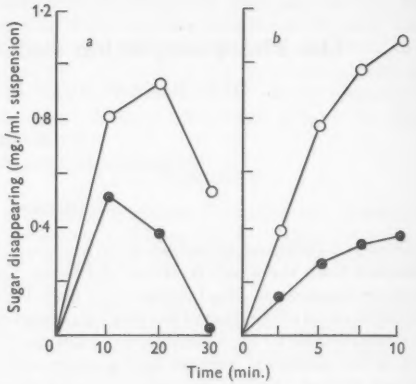


Fig. 1. Sugar disappearance as a measure of the hexokinase reaction. Each pair of curves was obtained from one animal. The test system employed is described in the experimental section. The glucose, ○—○, and fructose, ●—●, concentrations were 0·0055 M, and the ATP concentration was 0·033 M.

ATP (0·033 M) gave a small reducing blank equivalent to about 0·01 mg. glucose on each estimation, and did not alter on incubation with the suspensions in absence of sugar (three suspensions).

Buffer solutions. Three suspensions were tested for hexokinase activity with both glucose and fructose (0·0055 M) in the following buffers: veronal-acetate buffer (pH 8·0, 0·05 M), phosphate buffer (pH 8·0, 0·05 M), and bicarbonate buffer (pH 7·5, 0·2% $NaHCO_3$ with a gas mixture of 95% N_2 and 5% CO_2). The rates appropriate for each sugar did not differ with the buffer used. In glycine buffer (pH 8·5, 0·05 M) the rate with glucose was depressed relatively to the rate with fructose, and a slight increase of inorganic phosphate at the expense of 9 min.-labile P was observed. In borate buffer (pH 8·0, 0·025 M) phosphatase and ATPase activities were depressed by 50%, and lower concentrations of ATP could be used to give the same rates observed in veronal-acetate buffer at a higher ATP concentration.

Added magnesium. The suspension contained enough magnesium to allow some phosphorylation to occur, but the maximum rate was not obtained unless added $MgCl_2$ were present in a final concentration of 0·01 M.

Time of incubation. A fall in the reducing power of the samples was observed during the first 10 min., and the disappearance of sugar proceeded at a steady rate during the first 5 min. of the incubation period. After the first 10 min. the reducing power rose again (Fig. 1) but this could be prevented by the addition of more ATP, by the addition of fluoride to depress ATPase activity, or by the addition of pyruvate.

During the first 10 min. of the reaction the ratio between the phosphorylation rates of glucose and fructose remained the same (Table 1), but after this time the rate of the reaction with fructose fell off much more rapidly than that with glucose. This constancy of ratio during the early part of

Table 1. *Relative rates of phosphorylation of fructose and glucose*

(The concentrations of fructose and glucose were 0·0055 M, and that of ATP 0·033 M. Results are expressed as the mean ratio $\times 100 \pm$ S.D. (number of suspensions) of fructose to glucose disappearing (mg. sugar/ml. suspension) at progressive intervals.)

Time from start of incubation (min.)	Ratio of phosphorylation fructose/glucose $\times 100$
2·5	46±4·2 (19)
5	48±3·6 (25)
10	50±3·7 (49)
20	42±5·0 (12)
30	30±4·6 (12)

the incubation period was found with almost every one of the tissue suspensions examined, not only with glucose and fructose, but with mannose and xylose as well. With galactose, however, the rate of the reaction fell off sharply after the first 2·5 min.

Stability during incubation. Samples of suspensions were incubated at 30° for a given time in the presence or absence of sugar. These samples were then tested for hexokinase activity in the usual way (Table 2). The inactivating effect of previous incubation upon hexokinase activity and the stabilizing effect of added sugar during incubation were found to be very variable. The rate of the reaction with fructose and galactose fell considerably on incubation, and neither sugar showed any stabilizing effect. The rates with glucose, mannose and xylose were unaffected by incubation in the absence of sugar. ATP had no protective effect if added during incubation.

Concentration of tissue suspension. The rate of the hexokinase reaction with all five sugars was linear with increasing amount of suspension up to a concentration of 1 part suspension in 3 of the digest.

The phosphatase and ATPase reactions. Fig. 2 shows the appearance, over a period of time, of free sugar from glucose 1- and glucose 6-phosphates, from fructose 6-phosphate and from fructose 1:6-diphosphate. It also shows the appearance of inorganic phosphate from ATP. All monophosphates gave rise to free sugar at the same rate, but the diphosphate did so at only half the rate. The reaction with ATP ceased when both the 9 min.-labile P groups were converted into inorganic phosphate. The rate of appearance of free sugar increased with increasing concentration of monophosphate esters up to 0·01 M (Table 3). The rate of sugar liberation with the diphosphate was only half that observed with the monophosphates, and did not increase with concentrations above 0·007 M. The rate of appearance of inorganic phosphate from ATP increased up to an ATP concentration of 0·033 M. The inorganic phosphate appearing was equivalent to the 9 min.-labile P disappearing.

Both phosphatase and ATPase activity were raised 30% if $MgCl_2$ (final concentration 0·01 M) was added to the reaction mixture. Fluoride (0·06 M) abolished this effect. Phlorrhizin (0·004 M) and iodoacetate (0·008 M) did not affect phosphatase or ATPase activity. Pre-incubation of the suspension at 30°, under conditions similar to those used in the study of the hexokinase, had no effect upon either phosphatase or ATPase activity. The rate of both reactions increased proportionally to the concentration of the suspension, up to 1 part suspension in 3 of the digest.

Table 2. *The stability of the hexokinase on incubation*

(Samples of suspension were incubated (30°) at pH 8·0, under conditions of sugar concentration and incubation time shown below, and then tested at an ATP concentration of 0·033 M and a sugar concentration of 0·0055 M (glucose, galactose) or 0·02 M (fructose, mannose, xylose). The rate of the hexokinase reaction was expressed as 'mg. sugar disappearing/ml. suspension/2·5 min. (galactose) or 5 min. (other sugars)'. The results are given as: mean ± S.D. (number of suspensions).)

Sugar examined and time of incubation	Unincubated control	Incubated without sugar	Incubated with sugars (M)				
			0·0055	0·01	0·017	0·02	0·028
Glucose, 5 min.	0·5±0·07 (12)	0·47±0·09 (12)	0·61±0·04 (3)	0·56±0·12 (4)	0·57±0·01 (3)	—	—
Fructose, 5 min.	1·13±0·10 (13)	0·33±0·04 (13)	0·60±0·20 (3)	—	0·51±0·12 (5)	—	0·52±0·22 (3)
Fructose, 10 min.	1·39±0·03 (4)	0·38±0·03 (4)	0·51±0·05 (3)	0·37±0·05 (3)	0·30±0·13 (4)	0·50±0·07 (3)	0·51±0·14 (3)
Mannose, 5 min.	0·59±0·05 (5)	0·67±0·12 (5)	—	—	—	0·86±0·13 (5)	—
Xylose, 5 min.	0·34±0·05 (5)	0·41±0·04 (2)	—	—	—	0·41±0·03 (2)	—
Galactose, 2·5 min.	0·91±0·04 (3)	0·25±0·02 (3)	—	0·38±0·06 (3)	—	—	—

Table 3. *The effect of substrate concentration upon the rates of the phosphatase and ATPase reactions*

(The rates of the phosphatase reaction are expressed as 'mg. free sugar appearing/ml. suspension/5 min.'. The rates of the ATPase reaction are expressed as 'mg. inorganic phosphate appearing or 9 min.-labile P disappearing/ml. suspension/10 min.'. The results are given as: mean ± S.D. (no. of suspensions).)

Substance examined	Phosphorylated sugar (M) 0·0035	0·007	0·01	0·028
Phosphatase				
Glucose 6-phosphate*	0·48 ± 0·21 (8)	0·78 ± 0·04 (8)	0·99 ± 0·06 (8)	0·96 ± 0·05 (8)
Fructose 6-phosphate*	0·49 ± 0·03 (8)	0·84 ± 0·04 (8)	0·99 ± 0·05 (8)	0·90 ± 0·03 (8)
Glucose 1-phosphate	0·49 ± 0·04 (3)	0·80 ± 0·11 (3)	1·03 ± 0·07 (3)	1·14 ± 0·05 (3)
Fructose 1:6-diphosphate	0·29 ± 0·03 (3)	0·49 ± 0·03 (3)	0·48 ± 0·00 (3)	0·73 ± 0·00 (3)
	ATP concentration (M) 0·006	0·013	0·022	0·033
Adenosine triphosphatase				
Appearance of inorganic phosphate	0·52 ± 0·05 (4)	0·81 ± 0·06 (4)	0·94 ± 0·04 (3)	1·05 ± 0·12 (4)
Disappearance of 9 min.-labile P	0·41 ± 0·06 (4)	0·73 ± 0·04 (4)	0·81 ± 0·07 (4)	0·98 ± 0·07 (4)

* Same suspension used.

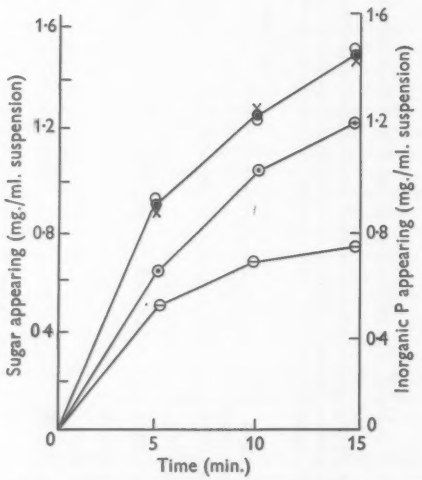


Fig. 2. Phosphatase and ATPase activity. One animal was employed, using a test system as described in the experimental section. The concentration of phosphorylated sugars employed was 0·028 M, and of ATP 0·033 M. Glucose 6-phosphate, ○—○; fructose 6-phosphate, ●—●; glucose 1-phosphate, ×—×; fructose 1:6-diphosphate, ⊖—⊖; ATP, ⊙—⊙.

The effect of ATP concentration upon the rate of the hexokinase reaction

Glucose and fructose. The rate of the hexokinase reaction at 0·0055 M glucose increased with the ATP concentration (0·013–0·04 M), whilst the rate with 0·0055 M fructose was unaffected by changes in ATP concentration over the same range (Table 4). At an ATP concentration of 0·013 M the rates with both sugars were the same, whilst at 0·033 M ATP the rate with glucose was nearly twice the rate observed with fructose. When, however, the fructose concentration was raised to 0·028 M the rate of the reaction varied with the ATP concentration in a manner similar to that observed with a glucose concentration of 0·0055 M.

Mannose and xylose. At a sugar concentration of 0·0055 M the rate of the hexokinase reaction with mannose and xylose was not appreciably affected by changes in ATP concentration (Table 4). At a sugar concentration of 0·022 M the rate varied more markedly with the ATP concentration.

Galactose. At a sugar concentration of 0·0055 M the rate of the phosphorylation reaction varied with the ATP concentration over the range 0·013–0·033 M (Table 4).

The effect of sugar concentration upon the rate of the hexokinase reaction

Glucose and fructose. The rate of the reaction with glucose, at a constant ATP concentration of 0·033 M, showed a sharp drop with increasing glucose concentration, this drop being usually observed at 0·016 M glucose. With a further increase in the concentration the rate rose sharply, until at a concentration of 0·028 M glucose the rate was the same as that obtained at 0·0055 M glucose (Table 5). Out of twenty-five suspensions examined twenty-two showed this sharp minimum. Increasing the ATP concentration to 0·052 M led to the flattening of this dip in the glucose concentration curve, although the rate at 0·028 M or 0·0055 M glucose was unaffected. The rate of the hexokinase reaction varied with the glucose concentration over the range 0·0014–0·0042 M, at an ATP concentration of 0·033 M (Table 6). In contrast with the reaction with glucose, the rate of the reaction with fructose at a constant ATP concentration of 0·033 M increased steadily with the fructose concentration to 0·022 M. The rate at the higher fructose concentration was not increased appreciably by raising the ATP concentration.

Mannose and xylose (Table 5). The rate of the reaction with mannose and xylose increased with sugar concentration from 0·0055 to 0·022 M. The phosphorylation rate of mannose was a little greater than that of xylose, the rates with both sugars being less than a third of the rate observed with glucose at a sugar concentration of 0·0055 M. At a sugar concentration of 0·028 M the rates, especially with mannose, approached that observed with glucose at this concentration.

Table 4. *The effect of ATP concentration upon the rate of the hexokinase reaction*

(The sugars were tested at the sugar concentrations indicated. The rates of the reactions are expressed as 'mg. sugar disappearing/ml. suspension/10 min. (2·5 min. for galactose)'. The results are given as: mean ± S.D. (number of suspensions), extreme range of values.)

Sugar examined (M)	ATP concentration (M)					
	0·013	0·020	0·026	0·033	0·040	0·052
Glucose (0·0055)	0·52 ± 0·05 (10) 0·24–0·66	0·64 ± 0·03 (10) 0·39–0·78	0·88 ± 0·04 (9) 0·72–1·02	1·08 ± 0·06 (7) 0·81–1·44	1·09 ± 0·06 (7) 0·96–1·50	— —
Fructose (0·0055)	0·48 ± 0·04 (10) 0·33–0·69	0·60 ± 0·04 (9) 0·42–0·63	0·58 ± 0·03 (9) 0·42–0·72	0·60 ± 0·04 (7) 0·42–0·75	0·58 ± 0·02 (7) 0·45–0·69	— —
Fructose (0·028)	0·60 ± 0·03 (3) 0·42–0·78	0·96 ± 0·05 (3) 0·84–1·44	1·23 ± 0·08 (3) 1·01–1·48	1·46 ± 0·08 (3) 1·42–1·56	1·80 ± 0·00 (3) 1·80–1·80	1·79 ± 0·06 (3) 1·75–1·84
Mannose (0·0055)	— —	0·26 ± 0·07 (4) 0·09–0·45	0·34 ± 0·05 (4) 0·24–0·45	0·39 ± 0·05 (3) 0·27–0·51	0·38 ± 0·06 (4) 0·15–0·51	— —
Mannose (0·022)	0·19 ± 0·01 (4) 0·18–0·21	— —	0·63 ± 0·02 (4) 0·54–0·72	0·84 ± 0·03 (4) 0·77–0·90	0·64 ± 0·12 (4) 0·24–0·90	— —
Xylose (0·0055)	— —	0·12 ± 0·05 (4) 0·03–0·21	0·23 ± 0·04 (3) 0·15–0·33	0·26 ± 0·03 (4) 0·18–0·33	0·24 ± 0·04 (4) 0·15–0·33	— —
Xylose (0·022)	0·16 ± 0·02 (4) 0·12–0·21	— —	0·51 ± 0·03 (4) 0·42–0·57	0·64 ± 0·05 (4) 0·54–0·81	0·51 ± 0·02 (4) 0·45–0·54	— —
Galactose (0·0055)	0·15, 0·30 (2)	0·30, 0·60 (2)	0·54, 0·66 (2)	0·84, 0·96 (2)	0·84, 1·02 (2)	—

Table 5. *The effect of sugar concentration upon the rate of the hexokinase reaction*

(The sugars were tested at the ATP concentration indicated. The rates of the reactions are expressed as: 'mg. sugar disappearing/ml. suspension/10 min.'. The results are given as: mean ± S.D. (number of suspensions), extreme range of values.)

	Sugar concentration (M)				
	0·0055	0·011	0·016	0·022	0·028
Glucose + 0·033 M ATP	1·07 ± 0·08 (12) 0·84–1·56	0·71 ± 0·09 (11) 0·36–1·20	0·35 ± 0·05 (12) 0·00–0·54	0·88 ± 0·06 (10) 0·54–1·14	0·97 ± 0·10 (10) 0·45–1·23
Glucose + 0·052 M ATP	1·09 ± 0·06 (4) 0·96–1·27	0·90 ± 0·09 (4) 0·60–1·02	0·94 ± 0·08 (4) 0·78–1·20	1·09 ± 0·03 (4) 1·02–1·20	1·07 ± 0·05 (4) 0·99–1·23
Fructose + 0·033 M ATP	0·63 ± 0·15 (8) 0·42–0·87	0·92 ± 0·05 (7) 0·63–0·90	1·24 ± 0·07 (8) 0·54–1·53	1·41 ± 0·14 (6) 1·08–1·92	1·54 ± 0·18 (6) 0·87–1·92
Mannose + 0·033 M ATP	0·30 ± 0·05 (5) 0·24–0·42	0·49 ± 0·04 (4) 0·36–0·63	0·73 ± 0·07 (5) 0·48–0·84	0·87 ± 0·05 (5) 0·72–0·96	0·84 ± 0·04 (4) 0·72–0·96
Xylose + 0·033 M ATP	0·28 ± 0·04 (5) 0·15–0·36	0·41 ± 0·06 (4) 0·21–0·54	0·59 ± 0·08 (5) 0·36–0·84	0·68 ± 0·05 (5) 0·36–0·84	0·63 ± 0·05 (4) 0·56–0·72
Galactose + 0·033 M ATP, incubated 2·5 min.	0·82 ± 0·04 (6) 0·63–0·90	0·17 ± 0·04 (6) 0·00–0·30	0·79 ± 0·10 (6) 0·36–1·02	0·83 ± 0·03 (6) 0·72–0·96	0·77 ± 0·05 (6) 0·48–0·96

Table 6. *The effect of low glucose concentrations upon the rate of the hexokinase reaction*

(The ATP concentration was 0·033 M. The rates of the reaction are expressed as: 'mg. sugar disappearing/ml. suspension/5 min.'. The results are given as: mean ± S.D. (number of suspensions), extreme range of values.)

Glucose concentration (M)			
0·0014	0·0028	0·0042	0·0055
0·39 ± 0·04 (5) 0·24–0·48	0·61 ± 0·04 (5) 0·54–0·72	0·75 ± 0·03 (4) 0·69–0·81	0·80 ± 0·04 (5) 0·75–0·96

Table 7. *The phosphorylation of sugar mixtures*

(The rates of the reactions are expressed as 'mg. sugar disappearing/ml. suspension/given unit of time'. The results are given as: mean ± S.D. (number of suspensions), extreme range of values.)

Mixture used	Single sugar, 0·0055 M, and ATP, 0·033 M		Mixture of two sugars, each 0·0055 M, and:	
Glucose/fructose (10 min.)	Glucose	Fructose	ATP, 0·033 M	ATP, 0·052 M
	1·06 ± 0·05 (9) 0·84–1·32	0·56 ± 0·04 (9) 0·42–0·72	0·69 ± 0·05 (9) 0·42–1·05	0·98 ± 0·14 (5) 0·72–1·32
Glucose/mannose (10 min.)	Glucose	Mannose		
	0·93 ± 0·05 (4) 0·78–1·02	0·32 ± 0·05 (4) 0·24–0·36	0·26 ± 0·08 (4) 0·03–0·45	0·38 ± 0·08 (4) 0·21–0·60
Glucose/xylose (5 min.)	Glucose	Xylose		
	0·96, 1·38 (2)	0·36, 0·36 (2)	0·18, 0·12 (2)	0·42, 0·48 (2)
Glucose/galactose (2·5 min.)	Glucose	Galactose		
	0·64 ± 0·06 (3) 0·54–0·78	0·80 ± 0·01 (3) 0·78–0·84	0·23 ± 0·12 (3) 0·00–0·27	0·69 ± 0·02 (3) 0·66–0·72

Galactose. The rate of galactose disappearance varied with increasing galactose concentration over the range 0·0055–0·028 M, in a manner similar to that observed with glucose over the same range, at the same ATP concentration, 0·033 M.

The phosphorylation of sugar mixtures (Table 7). Mixtures of glucose and of one other sugar, each at a concentration of 0·0055 M, ATP 0·033 M, did not show a rate of sugar disappearance equal to the sum of the rates observed when the sugars were tested separately at the same substrate concentrations. The rate with the mixture was usually lower than that observed with the glucose control, and was in some cases lower than the control with the other sugar. Raising the ATP concentration from 0·033 to 0·052 M resulted in an increase in the rate of sugar disappearance, although a rate equal to the sum of the control rates was never obtained.

DISCUSSION

A possible source of error in the determination of hexokinase activity could lie in the action of the phosphatase, releasing reducing substance from phosphorylated derivatives formed during the hexokinase reaction. The apparent difference observed between the phosphorylation rates of two sugars might thus be due to a difference in the rate of breakdown of their phosphorylated derivatives. However, glucose and fructose monophosphates showed the same dephosphorylation rate. Moreover, since the rate of the phosphatase reaction is dependent upon the rate of formation of phosphorylated sugar by the hexokinase, it follows that the rate of the hexokinase reaction must play a part in deciding the dephosphorylation rate. In the case of glucose, it can be calculated that the observed hexokinase activity during the first 5 min. of the incubation period is at least 70 % of what it should be in the absence of complicating phosphatase activity. Later on during the incubation period the ATP concentration falls, the hexokinase reaction slows down and the dephosphorylation reaction becomes more rapid than the hexokinase reaction, but this can be prevented by increasing the ATP concentration, or by depressing the ATPase and phosphatase activity by the use of fluoride or borate. It would appear that the observed behaviour of the hexokinase during the first 10 min. of the reaction period reflects the intrinsic properties of the enzyme, and is not an artifact.

The hexokinase of the intestinal mucosa shows two different patterns in its response to changes in sugar concentration. With fructose, mannose and xylose the phosphorylation rate varies with the sugar concentration over the range 0·0055–0·028 M. With glucose and galactose the rate falls to a sharp minimum at sugar concentrations in the intermediate part of this range, the rate rising again as a concentration of 0·028 M is approached, at which concentration the rate observed is the same as that observed at 0·0055 M. This falling off in rate can be partially prevented by raising the ATP concentration, although no effect is observed upon the rate of the reaction at the two limits of this sugar concentration range. The cause of the sharp drop in the rate of the hexokinase reaction at intermediate sugar concentrations is not known. Possibly two hexokinases might be concerned, each having different affinities for glucose, the affinity being determined by the ATP concentration. The work of Meyerhof & Geliazkowa (1947) shows that the affinity of brain hexokinase for glucose and fructose may be determined by the ATP concentration, but there is no evidence, outside the 'minimum' shown in the glucose and galactose concentration curves, that the hexokinase of the intestinal mucosa behaves in a similar manner. The data presented here with regard to the effect of ATP concentration on sugar utilization may be interpreted upon the assumption that

the intestinal hexokinase has different affinities for different sugars, which are unaffected by changes in the ATP concentration. As long as the sugar concentration limits the rate of the reaction, changes in ATP concentration will have a negligible effect upon the reaction rate, but as soon as the sugar concentration is raised to non-limiting levels the effect of changes in ATP concentration is noticeable.

The depression of phosphorylation rates observed with the intestinal hexokinase when mixtures of sugars are used broadly resembles the findings of Slein, Cori & Cori (1950) upon the phosphorylation of sugar mixtures by yeast hexokinase. These workers showed that in mixtures of fructose with either glucose or mannose the aldose suppressed the utilization of the ketose, whilst in a glucose-mannose mixture the rates with both sugars were depressed. With the intestinal hexokinase, all sugar mixtures examined gave a depression in rate, which suggests that interference takes place. In the case of the glucose-fructose mixture the depression is not so great as with some of the other mixtures, and it is possible that in this case the ATP concentration becomes the limiting factor. It is also possible that the addition of high concentrations of ATP could alter the affinity of the hexokinase for the sugars in the mixtures, as suggested by Slein *et al.* (1950).

The possibility exists that two or more hexokinases may be present in the intestinal mucosa. The separate existence of a glucokinase and a fructokinase has been demonstrated for brain (Meyerhof & Geliazkowa, 1947), muscle (Slein *et al.* 1950), and liver (Cori, Ochoa, Slein & Cori, 1951).

Recently Long (1952) has published data on glucose phosphorylation by dispersions of intestinal mucosa. His figures for $Q_{glucose}$, when converted into terms of mg. glucose disappearing/ml. suspension/5 min., yield an average value of 0·54 mg., with a range of 0·41–0·82 for eleven rats. These figures are a little lower than those given in the present study. The rate of the hexokinase reaction described by Long is unaffected by changes in glucose concentration over the range 0·0012–0·005 M, thus differing from the hexokinase discussed here, but otherwise the results of the two investigations are in agreement.

SUMMARY

1. The hexokinase reaction, the dephosphorylation of hexose phosphates, and adenosine triphosphatase activity have been studied in dispersions of intestinal mucosa.

2. Different sugars are phosphorylated at different rates, and the hexokinase shows different patterns in the response of the phosphorylation rates to changes in sugar concentration.

3. When the sugar concentration is not the limiting factor the rate with all the sugars examined varies in the same manner with the adenosine triphosphate concentration.

4. Sugars in mixtures show a depression in phosphorylation rate below that of single sugar controls.

5. The difference in the observed phosphorylation rates of glucose and fructose is due to a property of the hexokinase, not to a difference in the rate of liberation of free sugar from the phosphorylated sugars formed, nor to the conditions of assay.

I am very grateful to Dr A. M. Barrett for histological examination of specimens of intestinal mucosa.

REFERENCES

Bailey, K. (1949). *Biochem. J.* **45**, 479.
Beck, L. V. (1942). *J. biol. Chem.* **143**, 403.
Cori, C. F. (1925). *J. biol. Chem.* **66**, 691.
Cori, G. T., Ochoa, S., Slein, M. W. & Cori, C. F. (1951). *Biochim. biophys. Acta*, **7**, 304.
Davidson, J. N. & Garry, R. C. (1940). *J. Physiol.* **97**, 509.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Kjerulf-Jensen, K. (1942). *Acta physiol. scand.* **4**, 224.
Laszt, L. & Sullmann, H. (1935). *Biochem. Z.* **278**, 401.
Long, C. (1952). *Biochem. J.* **50**, 407.
Lundsgaard, E. (1933). *Biochem. Z.* **264**, 209, 221.
Lundsgaard, E. (1939). *Hoppe-Seyl. Z.* **261**, 193.
Meyerhof, O. & Geliazkowa, N. (1947). *Arch. Biochem.* **12**, 405.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Potter, V. R. (1947). *J. biol. Chem.* **169**, 17.
Slein, M. W., Cori, G. T. & Cori, C. F. (1950). *J. biol. Chem.* **186**, 763.
Verzár, F. & McDougall, E. J. (1936). *Absorption from the Intestine*, 1st ed. London: Longmans Green and Co.
Verzár, F. & Sullmann, H. (1937). *Biochem. Z.* **289**, 323.
Waring, W. S. & Werkman, C. H. (1942). *Arch. Biochem.* **1**, 303.
Wertheimer, E. (1933). *Pflüg. Arch. ges. Physiol.* **233**, 514.
Wilbrandt, W. & Laszt, L. (1933). *Biochem. Z.* **259**, 398.

The Phosphorylation and Absorption of Sugars in the Rat

2. SUGAR ABSORPTION *IN VIVO*, AND ITS RELATIONSHIP TO THE PHOSPHORYLATION OF SUGAR *IN VITRO*

By M. P. HELE*
Biochemical Laboratory, University of Cambridge

(*Received* 14 *January* 1953)

These experiments are intended to demonstrate that the rats of the stock used in the experiments on hexokinase activity (Hele, 1953) showed the same physiological behaviour as regards sugar absorption as animals described in the literature. Some information upon the rate of the hexokinase reaction catalysed by mucosa from the upper and lower halves of the intestine is also included.

EXPERIMENTAL

Sugar absorption in vivo. Sugar absorption was examined by the method of Cordier & Touzé (1948). The animals, of about 150 g. weight, were fasted for 48 hr. before the experiment. These animals, under ether anaesthesia, received an injection of 2 ml. of an isotonic sugar solution into the duodenum, and were killed by decapitation at intervals up to 1 hr. after the injection of the sugar. The small intestine was removed between ligatures, and its contents washed out as described by Cordier & Touzé. The washings were cleared of debris by boiling for 3 min. with 10 ml. of 0·1M zinc sulphate and 10 ml. of 0·2N sodium hydroxide, then filtering hot at the pump. The filtrate was made up to 500 ml., and the sugar content estimated by the method of Nelson (1944). It was observed during the course of these experiments that most of the sugar was absorbed from the upper quarter of the intestine.

The hexokinase reaction. The methods used were as described before (Hele, 1953). The intestine was cut into halves before weighing, and each half was weighed and treated separately.

RESULTS

Sugar absorption

Control experiments. Five animals received an injection of isotonic sodium chloride into the duodenum, and were sacrificed after 1 hr. Four of these animals showed no reducing substances in the intestinal washings, whilst one showed reducing substance equivalent to 1·5 mg. glucose.

The effect of time upon the rate of sugar absorption (*Fig.* 1). The animals used in these experiments were of similar, but not identical, stock with those used in the *in vitro* experiments. The rate of absorption of glucose and galactose remained linear with time until all the sugar was absorbed. The absorption rate with the other sugars fell off after 15–30 min., when only part of the sugar had disappeared from the intestine.

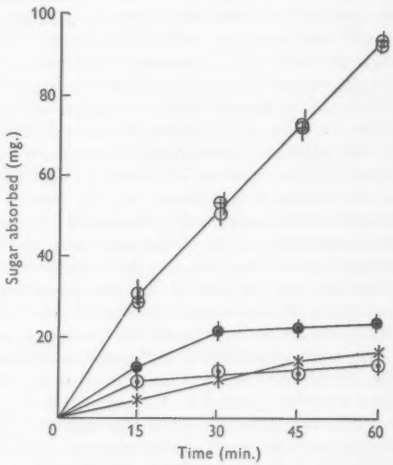


Fig. 1. Sugar absorption from rat intestine. The animals received an injection of 2 ml. of an isotonic sugar solution into the duodenum, and were killed at intervals up to 1 hr. ⊖—⊖, Galactose; ○—○, glucose; ●—●, fructose; ⊙—⊙, mannose; ×—×, xylose. The S.D. is indicated by vertical bars over each point.

Table 1. *Sugar absorption in the same stock of rats as those used for the studies on the hexokinase reaction*

(Values are expressed as mg. sugar absorbed in 30 min. after intraduodenal injection of 2 ml. isotonic sugar solution. The results are given as: mean ± S.D. (number of animals), extreme range of values.)

Galactose	Glucose	Fructose	Mannose	Xylose
64 ± 6 (5)	57 ± 4 (5)	28 ± 3 (5)	14 ± 3 (5)	15 ± 5 (5)
58–72	50–64	22–33	10–19	12–18

The absorption rate ratio and the phosphorylation rate ratio. As only a limited number of rats of the same stock as those used in the *in vitro* experiments was available, absorption rate ratios could only be

* Benn W. Levy Student.

Table 2. *Phosphorylation and absorption rate ratios*

(The phosphorylation- or absorption-rate ratio is defined as follows:

$$\frac{\text{mg. sugar absorbed or phosphorylated}}{\text{mg. glucose absorbed or phosphorylated}} \times 100.$$

The absorption-rate ratios were calculated from the data given in Table 1 and Fig. 1. The phosphorylation-rate ratios were calculated from the data of Hele (1953) relating to sugar phosphorylation at a sugar concentration of 0·0055 M, and an ATP concentration of 0·033 M.)

	Galactose / Glucose	Glucose / Glucose	Fructose / Glucose	Mannose / Glucose	Xylose / Glucose
Phosphorylation-rate ratios	126	100	55	36	24
Absorption-rate ratios	112	100	52	25	26
Absorption-rate ratios: Cori (1925)	110	100	43	19	15
Wilbrandt & Laszt (1933)	115	100	45	33	30
Westenbrink (1936)	108	100	42	15	13

Table 3. *The hexokinase reaction in the upper and lower halves of the small intestine*

(The rate of the reaction was measured after 5 min. incubation, for all sugars, except galactose (2·5 min.). Concentrations were: glucose and galactose, 0·0055 M; fructose, mannose and xylose, 0·028 M; ATP, 0·033 M. The results are given as: mean ± S.D. (number of suspensions), extreme range of values.)

	Galactose	Glucose	Fructose	Mannose	Xylose
Upper half	0·86 ± 0·07 (5) 0·78–0·94	0·87 ± 0·10 (11) 0·72–1·02	0·97 ± 0·10 (12) 0·72–1·20	0·49 ± 0·07 (5) 0·24–0·60	0·26 ± 0·10 (5) 0·24–0·36
Lower half	0·11 ± 0·10 (5) 0·00–0·39	0·45 ± 0·12 (11) 0·21–0·75	0·48 ± 0·12 (12) 0·36–0·78	0·38 ± 0·09 (5) 0·21–0·48	0·39 ± 0·22 (5) 0·15–0·69

Table 4. *The total phosphorylating capacity of the mucosa*

(Hexokinase activity is expressed as 'mg. sugar disappearing/ml. suspension/5 min. (aqueous suspensions) or 2·5 min. (Ringer suspensions)'. The sugar concentration was 0·0055 M, the ATP concentration 0·033 M. The results are given as: mean ± S.D. (number of suspensions), extreme range of values. The values for total sugar phosphorylation in 24 hr. were corrected by applying a temperature coefficient for the hexokinase activity, $Q_{30-38} = 1{\cdot}41$ (Long, 1952), and by adding to all values 30%, to compensate for the interference of the phosphatase reaction in the hexokinase assay (Hele, 1953).)

	Aqueous suspensions	Ringer suspensions		
	Glucose	Glucose	Galactose	Fructose
Observed values:				
Body weight of rats (g.)	158 ± 8 (10) 140–170	147 ± 8 (10) 144–164	As for glucose —	— —
Suspension obtained (ml.)	13·5 ± 1·1 (10) 11·0–17·5	8·8 ± 1·1 (10) 8·0–12·0	As for glucose —	— —
Hexokinase activity	0·92 ± 0·12 (10) 0·75–1·14	0·64 ± 0·07 (10) 0·54–0·78	0·87 ± 0·07 (10) 0·75–1·02	0·32 ± 0·03 (10) 0·27–0·42
Calculated values:				
Sugar phosphorylated mg./total mucosa/1 hr.	146 120–185	132 115–166	178 158–216	68 58–89
Sugar phosphorylated g./total mucosa/24 hr.	3·5 2·9–4·4	3·2 2·8–4·0	4·3 3·8–5·2	1·6 1·4–2·1
Sugar phosphorylated g./total mucosa/24 hr., corrected	6·6 5·3–7·7	6·0 5·2–7·4	8·0 7·1–9·6	2·9 2·6–3·9

measured for one interval of time (30 min.) after the intraduodenal injection of the sugar solution. This represented a compromise between the point at which the rates with fructose, mannose, and xylose declined, and the point at which sufficient sugar had vanished from the intestine to give a significant decrease in sugar concentration (Table 1). Good correlation was obtained between the absorption-rate ratios of these animals, the absorption-rate ratios of the animals of related stock, the absorption-rate ratios given in the literature, and the phosphorylation-rate ratios obtained from the experiments on the hexokinase reaction (Table 2).

The hexokinase reaction

The rate of the hexokinase reaction in the upper and lower halves of the small intestine (*Table* 3). Glucose and fructose were phosphorylated by tissue dispersions, prepared from the upper half of the intestine, at twice the rate given by dispersions pre-

pared from the lower half, and at rates similar to those shown by a dispersion prepared from whole intestine. Probably more hexokinase was present in the tissue suspensions obtained from the upper part of the intestine than the standard test system could measure. The phosphorylation of galactose was virtually confined to the upper half of the intestine. Mannose and xylose were phosphorylated at comparable rates in either half.

The total phosphorylating capacity of the mucosa. It was of interest to calculate the amount of sugar that could be phosphorylated by the whole mucosa in a given period of time, and to compare this with the amount of sugar absorbed during the same interval of time. Histological examination showed that all the epithelium of the mucosa passed into the suspension during preparation (Hele, 1953). Therefore, the sugar phosphorylated by the total volume of the suspension should be an indication of the phosphorylating capacity of the total absorbing surface of the small intestine. Table 4 shows that the total phosphorylating capacity of the suspension is more than sufficient to account for the phosphorylation of all the sugar absorbed during the experiments *in vivo*. It will also account for the phosphorylation of at least 50% of the carbohydrate consumed by an intact 150 g. rat in 24 hr., which amounts to about 7·5 g., chiefly as starch (Hele, 1953). After correction of the data on phosphorylation, the rate of the hexokinase reaction is sufficient to account for the phosphorylation of 70–100% of the carbohydrate consumed by the intact animal.

DISCUSSION

A relationship has been shown to exist between the relative rates of phosphorylation of certain sugars *in vitro*, at a sugar concentration of 0·0055M, and between the relative rates of absorption of these sugars *in vivo* from isotonic sugar solutions (0·3M). Raising the sugar concentration two- to three-fold results in an increase in the phosphorylation rates of the more slowly phosphorylated sugars, fructose, mannose and xylose, until these rates are close to those obtained for glucose and galactose at the same increased sugar concentration. A similar effect upon sugar absorption *in vivo* was described by Verzár and his co-workers (cf. Verzár & McDougall, 1936). These workers did not observe any sharp 'minimum' in their 'glucose concentration curve', such as is found with the phosphorylation reaction *in vitro*, but this could be explained by the existence *in vivo* of sufficient ATP to suppress this phenomenon.

A further parallelism between events *in vivo* and *in vitro* is shown by the fact that sugars in mixtures interfere with each other's phosphorylation rates (Hele, 1953). A similar effect of sugars in mixtures has been observed in studies on absorption rates (Cori, 1926; Cajori & Karr, 1935; Sobotka & Reiner, 1930). The observation also reported here, that certain sugars are more rapidly phosphorylated by dispersions from the upper half of the small intestine than by those from the lower half, is paralleled by the observations of Westenbrink (1936), Verzár & Wirz (1937) and Lium & Florey (1939), who found that these sugars are absorbed more rapidly from the upper part of the small intestine of the rat than from the lower.

The figures for the total phosphorylating capacity of the epithelium of the small intestine show that there is sufficient hexokinase present to account for the phosphorylation of all of the carbohydrate (7·5 g.) consumed by a 150 g. rat under completely physiological conditions in 24 hr. The rate of absorption as measured here by the method of Cordier & Touzé (1948) is only one-third (2·4 g. glucose in 24 hr.) of that of the intact animal. This can be attributed to the utilization of only a part of the total absorbing surface of the intestine during these experiments. These experiments reflected correctly the absorption-rate ratios, but gave no indication as to the total absorbing capacity of the intestine under normal conditions.

This striking parallelism between events *in vivo* and *in vitro* exists, although there is a marked difference between the sugar concentrations used *in vivo* (0·3M) and *in vitro* (0·0055M). It would be expected that *in vivo*, at a sugar concentration of 0·3M, the phosphorylation rates of all sugars would be the same, and that their absorption rates would follow suit. This is not so, and it must therefore be assumed that some factor, possibly membrane permeability, intervenes, causing the sugar concentration at the enzyme surface to behave as though it were some fifty times less than the sugar concentration in the intestinal lumen.

The evidence presented here suggests that the hexokinase of the intestinal mucosa is an integral part of the absorption mechanism for sugars. The other part of this mechanism involves the dephosphorylation of the esters formed. Since all the sugar monophosphates examined here were dephosphorylated at the same rate, it is unlikely that the dephosphorylation rate plays a part in deciding the relative absorption rates of the various sugars. These relative rates of absorption are determined by the affinity of the hexokinase for the different sugars. The distinction drawn between 'active' and 'passive' absorption is no longer a real one, for both are brought about by the same type of phosphorylation reaction. The effect of phlorrhizin or iodoacetate upon sugar absorption, whereby 'active' absorption is converted into 'passive' absorption may simply be due to the lowering of the concentration of available ATP in the mucosa, and to the effect of this upon the ability of the hexokinase to react with different sugars.

SUMMARY

1. The stock of rats used in the experiments upon the hexokinase reaction *in vitro* show the same behaviour regarding the absorption of sugar *in vivo* as do the rats used in experiments upon absorption described in the literature.

2. The phosphorylation-rate ratios for the sugars investigated are very similar to their absorption-rate ratios.

3. The rate of the hexokinase reaction catalysed by the intestinal mucosa is more than sufficient to account for the phosphorylation of all the sugar absorbed during the experiments *in vivo*. It is sufficient to account for the phosphorylation of at least 50% of the carbohydrate consumed by a normal 150 g. rat during 24 hr., and for the phosphorylation of 70–100% of this carbohydrate after certain legitimate corrections have been applied to the figures for the hexokinase reaction.

The author is indebted to Prof. D. Cordier for the hospitality of the Laboratoire de Physiologie Générale at the University of Lyons. It is a pleasure to acknowledge the advice and encouragement of Dr D. M. Needham, F.R.S., and the financial assistance of a grant from the Medical Research Council, and of a studentship from the University of Cambridge.

REFERENCES

Cajori, F. A. & Karr, W. G. (1935). *J. biol. Chem.* **109**, p. xiv.
Cordier, D. & Touzé, M. (1948). *C.R. Soc. Biol., Paris*, **142**, 91.
Cori, C. F. (1925). *J. biol. Chem.* **66**, 691.
Cori, C. F. (1926). *Proc. Soc. exp. Biol., N.Y.*, **23**, 290.
Hele, M. P. (1953). *Biochem. J.* **55**, 857.
Lium, R. & Florey, H. W. (1939). *Quart. J. exp. Physiol.* **29**, 303.
Long, C. (1952). *Biochem. J.* **50**, 407.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Sobotka, H. & Reiner, M. (1930). *Proc. Soc. exp. Biol., N.Y.*, **27**, 576.
Verzár, F. & McDougall, E. J. (1936). *Absorption from the Intestine*, 1st ed. London: Longmans Green and Co.
Verzár, F. & Wirz, H. (1937). *Biochem. Z.* **292**, 174.
Westenbrink, H. G. K. (1936). *Arch. néerl. Physiol.* **21**, 18.
Wilbrandt, W. & Laszt, L. (1933). *Biochem. Z.* **259**, 398.

A Chromatographic Study of Uroporphyrins from Cases of Cutaneous, Acute and Congenital Porphyria

By J. CANIVET* and C. RIMINGTON
Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1

(*Received* 28 *May* 1953)

Chromatographic methods (Nicholas & Rimington, 1949, 1951; Nicholas, 1951; Chu, Green & Chu, 1951; Falk & Benson, 1953*a*, *b*) have made possible the isolation and characterization of isomeric porphyrins. A study of porphyria cases has revealed much variety in the urinary porphyrin pattern (Nicholas & Rimington, 1951; Rimington & Miles, 1951; Macgregor, Nicholas & Rimington, 1952), but final agreement has not yet been reached as to which particular isomer predominates in the different kinds of porphyria. Thus, while Waldenström (1934, 1935), Waldenström, Fink & Hoerburger (1935) and Mertens (1936, 1937) independently described the urinary porphyrin characteristic of acute porphyria as uroporphyrin III, Grinstein, Schwartz & Watson (1945) and Watson, Schwartz & Hawkinson (1945) considered that the 'Waldenström porphyrin' consisted only of uroporphyrin I together with smaller quantities of a heptacarboxylic porphyrin. Although there appears to be a good deal of clinical and chemical variability between cases of acute porphyria, nevertheless Nicholas & Rimington (1953), employing a number of available techniques (Kennard & Rimington, 1953), have confirmed Waldenström's original conclusion in several cases of undoubted acute porphyria. Porphyria cutanea tarda (Waldenström, 1937), during exacerbations, may simulate acute porphyria closely, abdominal colic with or without nervous symptoms occurring and sometimes even porphobilinogen being found. However, the history of cutaneous lesions, presence of porphyrin in the plasma during the attack and high faecal porphyrin excretion during remission serve to distinguish it from acute porphyria. Some cases of porphyria, with cutaneous symptoms appearing late in life, seem to excrete uroporphyrin continuously in the urine (although perhaps with fluctuations of

* Chargé de Recherches à l'Institute National d'Hygiène, 3 rue Leon-Bonnat, Paris, 16e.

amount) and to manifest no abdominal or nervous symptoms. In many of these some degree of hepatic damage is present, e.g. from alcoholism, and it is difficult to decide whether such cases are examples of porphyria cutanea tarda which will later progress until the symptoms become more typical, or whether they should be placed in a separate category. Several such cases were available for study and are referred to provisionally as cases of 'cutaneous porphyria'.

It was felt that a full study of their urinary uroporphyrins would be of especial interest and might, perhaps, assist in their classification. For comparison in the present work, urinary uroporphyrins from cases of acute porphyria and congenital porphyria have been examined by the same methods.

EXPERIMENTAL

Materials

Cutaneous porphyria. The five cases, Ance., Vass., Turp., Hein., Fourn. with ages from 54 to 68 have been described by Bolgert, Canivet & LeSourd (1953). They were closely similar in all respects; they had bullous skin lesions but neither colics nor nervous symptoms and excreted large quantities of urinary uroporphyrin but no porphobilinogen (repeated tests). From urines of each of these patients, total porphyrin was adsorbed on talc at pH 3·5 and esterified with methanolic HCl. The esters were chromatographed on Al_2O_3 to separate the crude uroporphyrin fractions, which were then crystallized and their m.p.'s and rates of migration on paper determined (Table 1). These fractions were then combined and this material is hereafter referred to as 'pool uroporphyrin'; it was obtained from 38 l. of urine. From two further cases, Cam. and Clem., the urinary uroporphyrin was isolated individually. A further individual isolation was made from the urine of Ance.

Acute porphyria. The uroporphyrin fraction was similarly prepared from the urines of two cases, Jo. and Hem. Before adsorption on talc, the urines were heated at pH 4·2 for 20 min. in a boiling-water bath to convert all porphobilinogen into porphyrin (Table 1).

Congenital porphyria. Two cases were available, Yve. and Jul. and their uroporphyrins were prepared in a similar manner to the pool uroporphyrin (Table 1).

Methods

Column chromatography. This was carried out according to Nicholas (1951) using MgO grade III or Al_2O_3 grade IV or V. With Al_2O_3, impurities were removed by C_6H_6:$CHCl_3$, 2:1, any metalloporphyrin present by 1:1, coproporphyrins by 1:2, pentacarboxylic porphyrin by 1:3 or 1:4 and hexa- or hepta-carboxylic porphyrin by 1:6–1:10 mixtures (all v/v); pure $CHCl_3$ eluted a porphyrin the methyl ester of which had m.p. 211–216° and which appears to be octacarboxylic, but uroporphyrin was only eluted by $CHCl_3$ containing methanol. This mixture tended to mobilize the dark layer at the top of the column, which always retained some porphyrin; therefore the uroporphyrin fraction was generally obtained by extrusion and cutting. The retained top layer usually contained portions of all the constituents of the original mixture. Difficulty of this kind was hardly ever encountered with MgO columns (from which, of course, the uroporphyrins are the first to be eluted). A re-esterification was usually advisable before proceeding with the working up of this fraction. Repeated chromatography was essential to achieve homogeneity of any fraction.

Table 1. *Origin and properties of uroporphyrins used in this investigation*

Case	No. of crystallizations ($CHCl_3$:MeOH)	M.p. of porphyrin ester (°)	Comparison by chromatography: No. of carboxyl groups (Nicholas & Rimington, 1949, 1951)	Comparison by chromatography: Types of porphyrin present (Chu *et al.* 1951)	Comparison by chromatography: Ratio of uroporphyrin I to uroporphyrin III (Falk & Benson, 1953*a*)*
Cutaneous porphyria					
Ance.					
(1st batch)	1	272–5	8	Uroporphyrin only	3:1†
(2nd batch)	1	267–75	8	Uroporphyrin only	—
Vass.	2	254–7	—	Uroporphyrin only	1:3
Turp.	2	273–80	—	Uroporphyrin only	4:1†
Hein.	2	273–9	—	Uroporphyrin only	4:1
Fourn.	1	260	—	Uroporphyrin with traces of coproporphyrins I and III	3:2†
Cam.	4	285	—	—	17:3
Clem.	4	282	8	Uroporphyrin only	17:3
Pool‡	3	271–4	—	—	—
Acute porphyria					
Jo.	2	256–7	8	Uroporphyrin only	1:4
Hem.	1	255–7	—	Uroporphyrin only	1:3
Congenital porphyria					
Yve.	3	281	—	—	19:1
Jul.	3	285	—	—	19:1

* Approximate proportions, estimated by comparison with chromatograms of known mixtures.
† Confirmed by X-ray diffraction pattern (Kennard & Rimington, 1953).
‡ Bulked material from first five cases (see text).

Lutidine filter-paper chromatography. This was carried out according to Nicholas & Rimington (1949, 1951) and served to control homogeneity with respect to the number of carboxyl groups and to indicate the type of porphyrin present in fractions collected from columns.

Paper-chromatographic separation of isomers. Coproporphyrins I and III were separated according to Chu *et al.* (1951) and uroporphyrins I and III according to Falk & Benson (1953*a*, *b*).

Absorption spectra. The positions of the α bands were measured in $CHCl_3$ solution by the Hartridge reversion spectroscope.

Melting points. These were observed on the electrically heated microscope stage.

Decarboxylation. This was performed by heating with 1% (w/v) HCl in sealed tubes at 180–190° for 3 hr.

RESULTS

Cutaneous porphyria

The first chromatography of the combined crude esters was made upon magnesium oxide, grade III, in chloroform. Elution with chloroform, then with a chloroform:methanol mixture (100:0·5–100:2, v/v) removed first a yellow, non-porphyrin pigment (discarded), then the porphyrin, and left a residue of brown-black material at the top of the column (discarded). The porphyrin esters, derived from porphyrins containing, 8, 6, 5 and 4 carboxyl groups, respectively, were redissolved in benzene and placed upon a column of aluminium oxide, grade V, and development was carried out with benzene: chloroform mixtures as indicated under Methods. Each eluate was evaporated to dryness, redissolved in chloroform and examined by the Hartridge reversion spectroscope and by lutidine paper chromatography. The findings are recorded in Fig. 1 which shows the progress of the fractionation. Bands from different columns, having the same characteristics, were occasionally mixed to facilitate operations.

Fig. 1 shows that the fractionation proceeded very regularly, there being always a good correlation between position on the column, α band and result of lutidine chromatography. The following materials were obtained:

Metalloporphyrin ($F_1+G_1+M_1+N_1+O_1$). Pink, spectrum in chloroform $\lambda_{max.}$ 562·6, 530·2 mμ.; not split by acids. This appears to be a complex with copper or another heavy metal and has not been further studied.

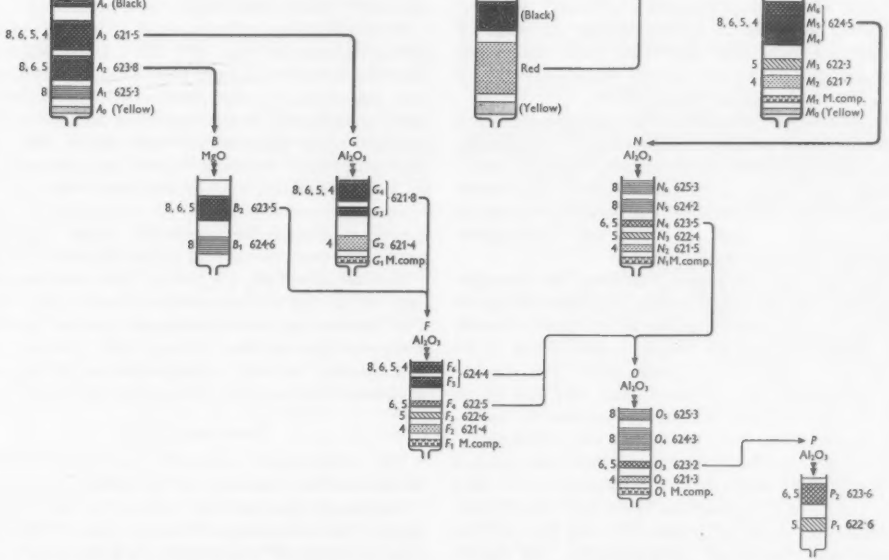


Fig. 1. Schematic representation of chromatographic separation of individual porphyrins from the crude fractions. 8, 6, 5, 4 = carboxyl numbers of lutidine chromatography; 621·5 = α band of spectrum in $CHCl_3$; M.comp. = metal complex.

Coproporphyrins (F_2, G_2, M_2, N_2, O_2). Paper chromatography (Chu *et al.* 1951) showed coproporphyrin I to predominate in F_2, G_2 and O_2 derived from the pool, whilst in M_2 and N_2 from case Ance. the series III isomer predominated. F_2, G_2 and O_2 were combined and examined; it had m.p. 243–246° and α band at 621·4 mμ. Lutidine chromatography showed that the porphyrin was tetracarboxylic, together with a trace of tricarboxylic porphyrin; isomer separation showed coproporphyrin I and a faint spot in the position of coproporphyrin III. M_2 and N_2 similarly afforded material of m.p. 136–140° and α band 621·6 mμ.; chromatography showed traces of tricarboxylic porphyrin and of series I isomer accompanying coproporphyrin III.

Pentacarboxylic porphyrin ($F_3+M_3+N_3+P_1$). In all these fractions, only pentacarboxylic porphyrin was revealed by lutidine chromatography. The material was dissolved in benzene, and the ester precipitated by addition of light petroleum; it crystallized from hot chloroform:methanol in loose aggregates of broad, curved needles, m.p. 210–214°, after recrystallization melting at 210–215°. This material, wt. 1·5 mg., had an α band at 622·5 mμ., which is typical of pentacarboxylic porphyrins. When chromatographed on paper by the dioxan method (Falk & Benson, 1953*a*), two spots formed: one, more intense, was in advance of the uroporphyrin III position, the other, less intense, was in advance of the uroporphyrin I position. Decarboxylation afforded a coproporphyrin the ester of which had m.p. 246–249° and was shown by chromatography to be mainly coproporphyrin I with a trace of coproporphyrin III.

Hexacarboxylic porphyrin. This was present in P_2, together with some pentacarboxylic porphyrin, from which it could not be entirely freed by chromatography upon aluminium oxide, magnesium oxide or calcium carbonate. The quantity was small and had an α band at 623·6 mμ. Attempted crystallization was unsuccessful.

Octacarboxylic porphyrin. During the development of columns *N* and *O* (Fig. 1), after the hexacarboxylic and pentacarboxylic porphyrin esters had been removed by benzene:chloroform (1:4–1:10, v/v), application of pure chloroform caused the separation of a distinct band (N_5 and O_4 in Fig. 1) which could be collected separately before the uroporphyrin ester, which was only mobilized by chloroform containing methanol. This material had α band 624·3–624·5 mμ. (cf. uroporphyrin ester 625·4 mμ.) and crystallized from hot chloroform:methanol in small aggregates of very fine needles somewhat resembling uroporphyrin III ester; m.p. 211–216°, unchanged by recrystallization from the same solvents, melting at 211–215° after crystallization from benzene:light petroleum and at 211–215° (remelting at the same temp.) when again recrystallized from chloroform:methanol.

Lutidine paper chromatography was carried out several times and invariably showed only one porphyrin, with R_F slightly greater than that of uroporphyrin but considerably less than would be expected for a heptacarboxylic porphyrin. That it was not uroporphyrin ester was clearly established by adding some of the material of m.p. 211–216° to uroporphyrin methyl ester and chromatographing the mixture upon aluminium oxide grade IV. Development with pure chloroform removed the ester of the new porphyrin in a compact band. It crystallized from chloroform:methanol in the usual form with m.p. 211–216° and its full visible spectrum, of aetio type, was 624·7; 571·9; 536·2; 503·9 mμ. The uroporphyrin ester which had been used in this mixture was recovered unchanged from the top of the column by elution with chloroform containing 1 % of ethanol.

Further information concerning the nature of this porphyrin ester was sought by subjecting it to a two-dimensional paper chromatography, the first solvents being those of Chu *et al.* (1951), the second the dioxan mixture of Falk & Benson (1953*a*). The porphyrin moved as a single spot, first like a uroporphyrin ester and then, with dioxan, to a position slightly in advance of uroporphyrin ester III. Chromatography together with a specimen of Watson's heptacarboxylic porphyrin methyl ester, m.p. 208°, clearly established their difference.

Decarboxylation. Of the total of 9·9 mg. of the porphyrin ester of m.p. 211–216°, 4 mg. was submitted to decarboxylation. The resultant porphyrins were transferred to ether, then to 0·1 N hydrochloric acid, precipitated, dried, esterified and chromatographed upon magnesium oxide grade III. The coproporphyrin fraction, eluted by chloroform:methanol (100:0·5, v/v) crystallized from ether in rosettes of needles, m.p. 150°, remelting at 176°; this is typical of coproporphyrin III. Only coproporphyrin III was revealed by paper chromatography.

Uroporphyrin ($A_1+N_6+O_5$). The ester crystallized from chloroform:methanol with m.p. 271–274°; this was subjected to decarboxylation, yielding a coproporphyrin ester of m.p. 220°. Paper chromatography showed coproporphyrin I together with smaller quantities of coproporphyrin III.

Acute porphyria

The uroporphyrin, isolated by similar methods to those described above from the urinary porphyrins of the case Jo., gave an ester of m.p. 256–257° and by paper chromatography (Falk & Benson, 1953*a*) consisted of about 80 % uroporphyrin III and 20 % uroporphyrin I; that from the case Hem. gave an ester of m.p. 255–257° and contained about 75 % of the series III isomer with 25 % of the series I (Table 1).

Congenital porphyria

The uroporphyrins from the two cases Yve. and Jul. yielded esters of m.p.s 281 and 285°, respectively. Assessment from the paper chromatograms indicated in each case about 95% uroporphyrin I and about 5% of uroporphyrin III. Decarboxylation of the Jul. specimen yielded coproporphyrin I, ester m.p. 248–251°.

DISCUSSION

The complexity of the urinary porphyrin pattern in these cases of 'cutaneous porphyria' resembles that found in acute porphyria (Nicholas & Rimington, 1951) congenital porphyria (Rimington & Miles, 1951) and some cases of porphyria cutanea tarda (Macgregor *et al.* 1952). In all the seven cases of cutaneous porphyria studied here, the uroporphyrin consisted predominantly of the I series isomer, although the proportion of I to III varied somewhat from case to case (Table 1). The melting point of the uroporphyrin ester mixture usually lay in the region of 270–280°, but in one instance (Fourn.) it was 260°. That this specimen, nevertheless, consisted mainly of uroporphyrin ester I was confirmed by its X-ray diffraction pattern (Kennard & Rimington, 1953); the low melting point is understandable by reference to the melting-point curve of mixtures of uroporphyrin methyl esters I and III established by Nicholas & Rimington (1953). In one case of the series (Vass.), a uroporphyrin was excreted of which the ester had m.p. 254–257° and which contained about 75% of uroporphyrin III and 25% of uroporphyrin I.

So far as the isomer type of the uroporphyrin excreted is concerned, the majority of these cutaneous porphyrias thus resemble cases of congenital porphyria more closely than they do cases of acute porphyria.

During the chromatographic operations, a fraction was obtained which was more easily eluted than uroporphyrin, could be separated from uroporphyrin in experimental mixtures, and yet which appeared to consist of octacarboxylic porphyrin only. It moved with an R_F slightly greater than that of uroporphyrin both as free porphyrin in the lutidine system and as ester in the dioxan system of Falk & Benson (1953*a*). Its ester had a constant melting point of 211–216° and decarboxylation yielded coproporphyrin III. The nature of this porphyrin remains obscure; it does not seem possible from chromatographic evidence that it can be a heptacarboxylic porphyrin and yet it is clearly different from both uroporphyrins I and III. We would emphasize that this new porphyrin has not been encountered on a single occasion only. It was isolated from different columns during the working up of the experimental material and has since been encountered in other cases of porphyria.

The pentacarboxylic porphyrin ester exhibits many points of interest. Whilst its α band at 622·5 mμ. is typical of that of other pentacarboxylic porphyrin esters which have been isolated (Grinstein *et al.* 1945; McSwiney, Nicholas & Prunty, 1950; Rimington & Miles, 1951), the melting point of this specimen is 210–215°, whereas the isomer yielding coproporphyrin I melts at 224° and that yielding coproporphyrin III melts at 180° (Watson, 1953). It would appear that in the present instance a mixture of pentacarboxylic porphyrins is present, since two spots were formed by dioxan paper chromatography and decarboxylation yielded both coproporphyrins I and III (the I series isomer predominating). It is quite clear that only by the use of these new chromatographic techniques and their further development can truly accurate knowledge be gained of the porphyrins present in porphyria urines.

SUMMARY

1. Chromatographic methods have been applied to the study of urinary uroporphyrins from seven cases of cutaneous porphyria. The patients, aged between 54 and 68, had suffered for 1–8 years from photosensitivity and bullous skin lesions. None excreted porphobilinogen or suffered from colics or nervous symptoms.

2. In six of these cases, the predominant isomer was uroporphyrin I (60–85%) but in one there was found approximately 75% of uroporphyrin III.

3. Urinary uroporphyrins from two cases of acute porphyria and two cases of congenital porphyria were similarly studied for comparison. The former contained 75–80% uroporphyrin III and 20–25% of uroporphyrin I, whilst in the congenital porphyria material about 95% of uroporphyrin I was found.

4. A porphyrin is described, the ester of which had m.p. 211–216°, and which behaved as an octacarboxylic porphyrin on lutidine paper chromatography, but with R_F very slightly greater than that of uroporphyrin, from which it is separable by column chromatography. On decarboxylation, it yielded coproporphyrin III.

5. A hexacarboxylic porphyrin was detected but not isolated.

6. A pentacarboxylic porphyrin was isolated, with ester of m.p. 210–214°. Paper chromatography by the dioxan method afforded two spots of unequal intensity and on decarboxylation coproporphyrin I was produced together with a smaller quantity of coproporphyrin III.

7. The relation of type of porphyria to isomeric pattern of porphyrin excretion is discussed.

One of us (J.C.) wishes to thank the Ciba Foundation for a grant which enabled him to pursue this study in England. Dr Bolgert kindly referred some of the patients to J.C. for biochemical study. A generous grant to C.R. from the Nuffield Foundation has made possible the establishment of a Unit for the Investigation of Pyrrole Pigment Metabolism.

Our thanks are due to Miss A. Benson for skilled assistance with paper chromatography, and to Mr V. K. Asta for preparing the figure.

REFERENCES

Bolgert, M., Canivet, J. & LeSourd, M. (1953). *Sem. Hôp. Paris*, **29**, 1587.
Chu, T. C., Green, A. A. & Chu, E. J. (1951). *J. biol. Chem.* **190**, 643.
Falk, J. E. & Benson, A. (1953*a*). *Biochem. J.* **53**, xxxii.
Falk, J. E. & Benson, A. (1953*b*). *Biochem. J.* **55**, 101.
Grinstein, M., Schwartz, S. & Watson, C. J. (1945). *J. biol. Chem.* **157**, 323.
Kennard, O. & Rimington, C. (1953). *Biochem. J.* **55**, 105.
Macgregor, A., Nicholas, R. E. H. & Rimington, C. (1952). *Arch. intern. Med.* **90**, 483.
McSwiney, R. R., Nicholas, R. E. H. & Prunty, F. T. G. (1950). *Biochem. J.* **46**, 147.
Mertens, E. (1936). *Hoppe-Seyl. Z.* **238**, i.
Mertens, E. (1937). *Hoppe-Seyl. Z.* **250**, 57.
Nicholas, R. E. H. (1951). *Biochem. J.* **48**, 309.
Nicholas, R. E. H. & Rimington, C. (1949). *Scand. J. clin. Lab. Invest.* **1**, 12.
Nicholas, R. E. H. & Rimington, C. (1951). *Biochem. J.* **48**, 306.
Nicholas, R. E. H. & Rimington, C. (1953). *Biochem. J.* **55**, 109.
Rimington, C. & Miles, P. A. (1951). *Biochem. J.* **50**, 202.
Waldenström, J. (1934). *Acta med. scand.* **83**, 281.
Waldenström, J. (1935). *Dtsch. Arch. klin. Med.* **178**, 38.
Waldenström, J. (1937). *Acta Med. scand. Suppl.* **82**.
Waldenström, J., Fink, H. & Hoerburger, W. (1935). *Hoppe-Seyl. Z.* **233**, 1.
Watson, C. J. (1953). Personal communication.
Watson, C. J., Schwartz, S. & Hawkinson, V. (1945). *J. biol. Chem.* **157**, 345.

The Chromatographic Separation of Free and Combined Plasma Cholesterol

By LYNDA M. H. KERR and W. S. BAULD
Department of Biochemistry, University of Edinburgh

(*Received* 8 *July* 1953)

The standard method of estimation of plasma cholesterol, free and combined (Schoenheimer & Sperry, 1934), involves the precipitation of cholesterol as the digitonide before and after hydrolysis. Separation and purification of free and esterified cholesterol in plasma extracts and direct estimation of the two fractions would greatly simplify the procedure. Trappe (1942) and Hess (1947) have demonstrated the ease with which the separation could be achieved by chromatography on activated alumina. Both workers, however, used eluents placed rather high in the series. These might be expected to elute other components of the plasma lipid fraction, thereby diminishing the effectiveness of the purification. Contamination of the cholesterol fractions was in fact noted by Trappe (1942). Re-investigation of the method of separation was desirable.

The problem of direct colorimetric estimation of the separated fractions was solved by Trappe (1942), by using a reaction in which esterification did not alter the colour production. This condition does not hold in the more commonly used Liebermann-Burchard procedure (see Sperry & Brand, 1943). For this reason Hess (1947) hydrolysed the esters before estimation. The separated fractions could, however, be directly estimated if the effect of esterification on the colour production were independent of the fatty acid group involved. The results of Yasuda (1936) suggested that this was the case and this approach to the problem was investigated further. It is known that cholesteryl esters in plasma are mainly derived from unsaturated acids, and recently Keegan & Gould (1953) have reported the isolation of cholesteryl oleate from plasma. Accordingly, both saturated and unsaturated esters were prepared and used as models.

METHODS

Purification of reagents

Chloroform. This was washed, dried over $CaCl_2$ and used freshly distilled.

Sulphuric acid. A.R. grade (British Drug Houses Ltd.).

Acetic anhydride. The A.R. grade was distilled at constant boiling point.

Alumina (Peter Spence). This was washed with water and methanol and dried to grade II activity (Brockmann & Schodder, 1941).

Light petroleum. A.R. grade, b.p. 60–80°.

Benzene. The A.R. grade was refluxed with conc. H_2SO_4 (30 ml./l.) for 30 min., washed with NaOH and water, dried over Na and distilled.

Acetone. This was refluxed with and distilled from $KMnO_4$, dried over K_2CO_3 and redistilled.

Ethanol. This was refluxed with and distilled from NaOH.

Standard solutions

Cholesterol, m.p. 148°; cholesteryl acetate, m.p. 114°; cholesteryl palmitate, m.p. 80°; cholesteryl stearate, m.p. 79°; cholesteryl oleate, m.p. 41°. (All compounds recrystallized to constant m.p.; all m.p. corr.) The anhydrous solid (20–30 mg., as cholesterol) was dissolved in 100 ml. $CHCl_3$.

Colour reactions

H_2SO_4-acetic anhydride reagent was prepared by adding, with mixing, 1 ml. H_2SO_4 to 20 ml. chilled acetic anhydride (at 0°) and again chilling for 10 min. It was used within 30 min. of preparation. Cholesterol, free and esterified, was estimated by the Liebermann-Burchard reaction. The cholesterol solution was evaporated to dryness and desiccated for at least 6 hr. over H_2SO_4 *in vacuo*. The residue was dissolved in 6 ml. $CHCl_3$, and 2 ml. H_2SO_4-acetic anhydride reagent were added with mixing. The colour was developed in the dark in a water bath at 25° for 10 min. and read within 5 min. in a Spekker photoelectric absorptiometer with Ilford filters no. 608 (690 mμ.).

Chromatography

All solvent proportions are given as % (v/v). Standard proportions were cholesterol 0·3–0·5 mg., Al_2O_3 200 mg. Chromatographic tubes were made from pipettes of 25 ml. capacity, cut through the bulb and having a stem 10–12 cm. long and 0·4 cm. int. diam. The Al_2O_3 was packed as a slurry in light petroleum (care was taken to exclude air) to a height of 3 cm. over a small plug of cotton wool in the tip of the pipette. The remaining part of the bulb and stem acted as a reservoir and was used to regulate the rate of flow to 20 ml./hr. Compounds applied to the column were dissolved in 0·5 ml. light petroleum and followed by three washes of 0·5 ml. of the same solvent. Unless otherwise stated, cuts of 5 ml. were taken.

Plasma extracts

The acetone-ethanol reagent, 1:1, was freshly prepared. The extraction was carried out by a slight modification of the method of Schoenheimer & Sperry (1934). Plasma (0·2 ml.) was pipetted into 3 ml. warm acetone-ethanol reagent in a 10 ml. glass-stoppered centrifuge tube. The solution was heated to boiling point in a water bath and shaken for 2 min. After cooling, the tube was centrifuged and the supernatant liquid transferred to a second tube. The residue was re-extracted with 2 ml. acetone-ethanol reagent as above, and the combined supernatant liquids were evaporated to dryness in a water bath in a current of air.

RESULTS AND DISCUSSION

The Liebermann-Burchard colour reaction

Because of the controversy in the literature about the optimum conditions for the Liebermann-Burchard reaction of cholesterol esters, the following experiments were carried out (cf. Sperry & Brand, 1943).

The effect of temperature on the rate of colour development. Known volumes of cholesterol and cholesteryl stearate solutions were prepared for estimation as described above. The colour which developed during incubation at 15, 20, 25 or 30° was read at 5 min. intervals. As is shown in Fig. 1, the colour developed by the ester reached its maximum more quickly than that of the alcohol, at all temperatures examined. At 15° the colour in both cases was slow to develop, and did not reach a maximum even after 20 min. incubation, while at 30° the colour was unstable, reached a maximum within 5 min. and faded almost immediately. Between 20 and 25°, however, the rate of colour development appeared to be independent of temperature. It reached a maximum after 10 min. incubation and remained constant for at least a further 10 min. These results are, in general, in agreement with those of previous workers, and other esters of cholesterol gave values similar to those of the stearate.

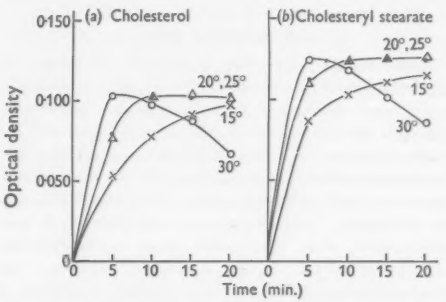


Fig. 1. The effect of temperature on the rate of colour development. (*a*) 200 μg. cholesterol were dissolved in 6 ml. $CHCl_3$ and 2 ml. acetic anhydride-H_2SO_4 mixture. The colour was read at time intervals after incubating at 15° (×—×), 20° (△—△), 25° (●—●), and 30° ○—○). (*b*) 304 μg. cholesteryl stearate (180 μg. cholesterol) were treated as (*a*).

The colour developed by cholesteryl esters. As is seen from Fig. 1, the intensity of colour developed by cholesteryl stearate is greater than that developed by an equivalent weight of the alcohol. The behaviour of other esters was therefore investigated. Samples of standard cholesteryl acetate, palmitate, stearate or oleate solutions were made up to 6 ml. with $CHCl_3$, 2 ml. of the H_2SO_4-acetic anhydride reagent were added, and the colour was developed in the dark at 25° for 10 min. The colour developed in each case was found to be independent of the esterifying acid and to be directly proportional to the cholesterol content of the sample. The colour produced by the esters is approximately 30 % more intense than that produced by cholesterol under identical conditions (see Table 1). Two standard

Table 1. *Colours produced by cholesterol and its esters*

(Conditions as in text, p. 873.)

Compound	Optical density: 200 μg. Cholesterol	Optical density: 400 μg. Cholesterol
Cholesterol	0·110	0·221
Cholesteryl acetate	0·141	0·286
Cholesteryl palmitate	0·144	0·283
Cholesteryl stearate	0·143	0·285
Cholesteryl oleate	0·139	0·280

curves are therefore required, one constructed from cholesterol and the other from any representative ester. Provided that allowance is made in this way for the enhancement of colour produced by esterification, it would appear that both cholesterol and its esters may be directly estimated by measurement of the intensity of the green colour developed when the Liebermann-Burchard reaction is allowed to proceed in the dark at 25° for 10 min.

The chromatographic separation of cholesterol and cholesteryl esters

Experiments with pure solutions. Mixtures of known amounts of cholesteryl stearate and cholesterol were applied in light petroleum to alumina columns as above and the light-petroleum eluates were examined for chromogenic material by the Liebermann-Burchard reaction. From this experiment and from others in which cholesteryl stearate or cholesterol were examined separately, it was established that the ester was quantitatively eluted by 20 ml. light petroleum, whereas the alcohol was retained even with 150 ml. Addition of benzene to the petroleum, to 50 %, resulted in the appearance of cholesterol, spread over several fractions. When the benzene was increased to 80 % the leading edge of the eluate pattern was much sharpened, and, as was to be expected from theoretical considerations (cf. Cassidy, 1951), the use of pure benzene gave a similar pattern. In a typical experiment 506 μg. cholesteryl stearate and 275 μg. cholesterol (equivalent to a total of 575 μg. cholesterol) were dissolved in light petroleum and added to an alumina column in the usual manner. The chromatogram was developed with 30 ml. light petroleum followed by 30 ml. benzene. 5 ml. cuts were taken and the Liebermann-Burchard colour was developed. The six light petroleum eluate fractions were found to contain 154, 75, 54, 22, 0 and 0 μg. cholesterol (as the stearate) and the six benzene fractions 17, 152, 76, 23, 0 and 0 μg. cholesterol, respectively. A standard procedure has therefore been adopted in which 30–40 ml. light petroleum are used as the first eluent and followed by a similar volume of pure benzene. Under these conditions all esters tested gave similar patterns.

It was suggested by Trappe (1940) that cholesterol might be chemically altered by contact with alumina, and there is also the possibility that the cholesteryl esters may be hydrolysed. The former possibility need not be considered under the present conditions in view of recovery figures (see later, Table 2). Cholesteryl acetate and stearate, chosen for their difference in chain length, were allowed to remain in contact with alumina on the column for 12 hr. before they were eluted with light petroleum and benzene in the usual manner. In the cases of both esters 98 % of the chromogenic material was recovered in the light petroleum fraction and none in the benzene, showing that no hydrolysis of the esters had occurred.

Experiments with plasma extracts. It was essential to determine whether or not the elution pattern of the cholesterol in acetone-ethanol extracts of plasma was the same as that of pure solutions. Plasma extracts were therefore applied to columns and eluted successively with light petroleum and benzene. Examination of the eluates by the Liebermann-Burchard reaction showed a pattern similar to that given by mixtures of cholesteryl stearate and cholesterol, i.e. two peaks, one in the first 20 ml. of light petroleum and the other in the first 20 ml. of benzene, representing the ester and the alcohol fractions, respectively. A plasma containing 210 mg. cholesterol/100 ml. was used in a typical experiment. The extract from 0·2 ml. plasma was applied to a column which was developed with light petroleum, 30 ml., followed by an equal volume of benzene. Twelve 5 ml. cuts were collected and the Liebermann-Burchard colour was developed in each. The light petroleum eluates contained 22, 180, 67, 50, 0 and 0 μg. cholesterol, and the benzene eluates 10, 35, 26, 15, 0 and 0 μg. cholesterol respectively, thus giving a value of 159 mg./100 ml. for the ester cholesterol and 43 mg./100 ml. for the free.

There was also the danger of eluting some substance in the plasma extract along with either the ester or free cholesterol fractions which, although non-chromogenic, might interfere with the colour reaction. Samples of standard solutions of cholesteryl stearate and cholesterol were added to plasma extracts of known cholesterol content, and the whole was chromatographed as for plasma extracts. The results are shown in Table 2. Cholesteryl stearate was recovered from 96 to 98 % in the light petroleum fraction and cholesterol from 93 to 97 % in the benzene. Moreover, no anomalous peaks were detected. In a few early experiments an ether-ethanol extract of plasma was used, but this was abandoned when it was discovered that substances, probably phospholipids, which interfered with the Liebermann-Burchard reaction were eluted by benzene. Unpurified A.R. benzene also contained an impurity which gave a green colour with the Liebermann-Burchard reagents.

Table 2. *Recoveries of added cholesteryl stearate and cholesterol from plasma extracts*

Cholesterol (mg./100 ml.)					
Amount in plasma extract		Amount added		Amount recovered	
Ester	Free	Ester	Free	Ester	Free
56	94	100	30	153	122
56	94	150	60	200	151
145	47	100	30	243	76
145	47	150	60	292	105
212	78	100	30	308	107
212	78	150	60	357	136

Table 3. *Comparison between the Schoenheimer-Sperry and the chromatographic methods*

(In the Schoenheimer-Sperry method, total and free values are obtained by estimation, and ester by difference. In the chromatographic method, ester and free values are obtained by estimation, and total by summation.)

Schoenheimer-Sperry. Cholesterol (mg./100 ml.)			Chromatographic. Cholesterol (mg./100 ml.)		
Total	Ester	Free	Total	Ester	Free
295	210	85	282	202	80
194	144	50	195	150	45
171	123	48	170	125	45
243	170	73	238	168	70
290	215	75	290	210	80
182	132	50	185	130	55
141	100	41	150	105	45
162	113	49	158	111	47
157	114	43	151	113	38
214	159	55	210	156	54
180	131	49	187	135	52
265	193	72	263	188	75
430	235	195	432	228	204
215	155	60	224	160	64
130	98	32	132	102	30

In many cases after applying the plasma extract to the column, a small residue, insoluble in light petroleum, was left behind. This was neglected as it was found to give no colour in the Liebermann-Burchard reaction. A narrow, yellow band was usually formed on the surface of the alumina, but this was not eluted by either solvent, all eluates being colourless.

In all routine experiments fractions of 20 ml., followed by two of 10 ml. each, were collected with each eluent. The final 10 ml. fraction was always a blank containing no chromogenic material. Thus even with plasma of abnormally high cholesterol content no ester can be carried into the benzene fraction and all the material is recovered.

Reproducibility of results. Twelve separate extracts of the same plasma were prepared, and the free and combined cholesterol determined as above. The average value for ester cholesterol was found to be 153 mg./100 ml. with a range of 143–162 mg./100 ml. and for free cholesterol 51 mg./100 ml. with a range of 45–57 mg./100 ml.

Comparison of the present method with the Schoenheimer-Sperry method. The applicability of this method for routine clinical estimation of plasma cholesterol was checked by comparing results obtained by the Schoenheimer-Sperry method and by chromatography using the same plasma (Table 3). The authors are grateful to Dr G. S. Boyd of this Department for allowing them to quote his results for the Schoenheimer-Sperry method and for the supply of plasma.

Although the accuracy of the present method is not as great as that of the Schoenheimer-Sperry method (1–2%), it would appear to be satisfactory for most clinical purposes.

The mean of the differences between the two methods is 4 mg./100 ml. in the case of total, ester, and free cholesterol; the ranges of the differences are 1–13, 1–8 and 1–9 mg., respectively.

SUMMARY

1. The chromatographic separation of cholesterol from its esters using alumina and a solvent system of light petroleum followed by benzene has been achieved.

2. It has been shown that esters of cholesterol may be estimated directly by the Liebermann-Burchard reaction.

3. On the basis of the above, a method has been developed for the direct determination of free and combined cholesterol in blood plasma.

One of the authors (L. M. H. K.) is indebted through the Rheumatic Unit, Northern General Hospital, Edinburgh, to the Nuffield Foundation and to the Medical Research Council for grants during the course of this work.

REFERENCES

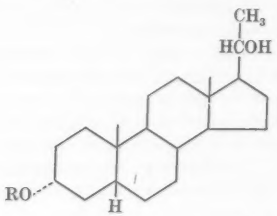
Brockmann, H. & Schodder, H. (1941). *Ber. dtsch. chem. Ges.* **74**, 73.
Cassidy, H. G. (1951). *Techniques of Organic Chemistry*, Vol. 5, p. 153. London: Interscience Publishers Ltd.
Hess, W. C. (1947). *J. Lab. clin. Med.* **32**, 1168.
Keegan, P. & Gould, R. G. (1953). *Fed. Proc.* **12**, 228.
Schoenheimer, R. & Sperry, W. H. (1934). *J. biol. Chem.* **106**, 745.
Sperry, W. H. & Brand, F. C. (1943). *J. biol. Chem.* **150**, 315.
Trappe, W. (1940). *Biochem. Z.* **307**, 97.
Trappe, W. (1942). *Hoppe-Seyl. Z.* **273**, 177.
Yasuda, M. (1936). *J. Biochem., Tokyo*, **24**, 443.

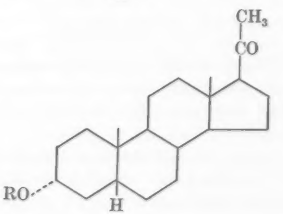
Oxidative Fission of Pregnanediol Glucuronide

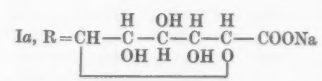
By J. K. Norymberski and Ann T. Sermin
Rheumatism Research Unit, Nether Edge Hospital, Sheffield, 11

(*Received* 17 *July* 1953)

Reactions likely to occur between urinary steroid conjugates and sodium bismuthate may be divided into two groups: (i) oxidative fission of 1:2-glycols, 1:2-ketols and α-hydroxyacids incorporated in the steroid carbon skeleton, and (ii) analogous reactions in the carbohydrate moiety of steroid glucuronides (or glucosiduronic acids*). Of the former reactions those concerning the corticosteroid side chain have been described in recent communications from this laboratory (Brooks & Norymberski, 1952, 1953; Norymberski, 1952; Norymberski, Stubbs & West, 1953). We now wish to report the oxidative cleavage by bismuthate of steroid glucosiduronic acids under conditions identical with those employed for the oxidation of corticosteroids. Part of this work has been briefly reported elsewhere (Norymberski, 1953).







Ib, R=CHO

Ic, R=H

IIb, R=CHO

IIc, R=H

Sodium pregnanediol glucuronidate of pregnancy urine (Venning, 1937, 1938)—a mixture consisting mainly of sodium 20α-hydroxypregnan-3α-yl glucopyranosiduronate (I*a*) and sodium 20-oxopregnan-3α-yl glucopyranosiduronate (pregnanolone glucuronide) (II*a*) (Sutherland & Marrian, 1947)—was treated with sodium bismuthate in aqueous acetic acid. The crude product was chromatographed on alumina: the four fractions which were further investigated gave, in order of elution, an oil which on prolonged contact with alumina yielded 3α-hydroxypregnan-20-one (II*c*), 3α-formoxypregnan-20α-ol (I*b*), 3α-hydroxypregnan-20-one (II*c*) and pregnane-3α:20α-diol (I*c*). The first fraction is considered to consist of impure 3α-formoxypregnan-20-one (II*b*) on the basis of its ready elution from alumina and its conversion into II*c*. 3α-Formoxypregnan-20α-ol (I*b*) was characterized by its deformylation on alumina to the diol I*c* and by conversion into 3α-hydroxypregnan-20-one (II*c*) on oxidation with chromic acid followed by hydrolysis on alumina. Deformylation on alumina has been described by Koechlin, Kritchevsky & Gallagher (1950) for 3α-formoxy-5β-steroids and by Levin, McIntosh, Spero, Rayman & Meinzer (1948) for 5:6-unsaturated 3β-formoxysteroids, whilst Steinegger & Katz (1947) found that 3β-formoxycholestane (5α) is unchanged by alumina.

The oxidative cleavage of a urinary glucosiduronic acid *in situ* was next investigated. Goldfine & Cohen (1953) have shown, by means of enzymic hydrolysis with β-glucuronidase, that pregnane-3α:20α-diol (I*c*) is excreted exclusively in

* Nomenclature as recommended by the Carbohydrate Nomenclature Sub-Committee of the Chemical Society (*J. chem. Soc.*, 1952, p. 5117): the term 'glucosiduronic acid' replaces 'glucuronide' for a glycoside in which the terminal CH_2OH has been oxidized to a carboxyl group. In the present paper 'pregnanediol glucuronide' and 'sodium pregnanediol glucuronidate' are considered trivial names and are retained unchanged.

Urine (Quarter of a 24-hr. specimen)	*A* Treated with $NaBiO_3$	*B* Hydrolysed with acid	*C* Untreated
↓ Ethyl acetate			
Crude extract	940 mg., dark gum	480 mg., dark gum	710 mg., dark gum
↓ Alkali			
Neutral extract	55 mg., partly crystalline	30 mg., partly crystalline	14 mg., gum
↓ Chromatography			
→Fraction 1	21 mg., gum	12 mg., gum	9 mg., gum
Fraction 2	15 mg., cryst.	12 mg., cryst.	2 mg., gum
↓ High vacuum 190°			
Sublimate	11 mg., oil and crystals*	9·7 mg., oil and crystals	Trace
↓ Recryst. from acetone			
Pure pregnane-3α:20α-diol	5·3 mg.	4·6 mg.	—

* At this stage an unidentified by-product was separated (see p. 878).

Fig. 1. Isolation of pregnane-3α:20α-diol from pregnancy urine.

conjugation with glucuronic acid. The quantity of the diol (I*c*) isolated from pregnancy urine after treatment with sodium bismuthate as compared with that obtained after acid hydrolysis should therefore indicate the efficacy of the oxidative procedure. The comparative experiments were carried out as shown in Fig. 1: oxidative or hydrolytic treatment gave pregnanediol (I*c*) in approximately the same yield. To ensure that no free diol was present in the urine specimen under investigation, the extraction of an untreated sample was carried out as well.

EXPERIMENTAL

Melting points were taken on a Kofler stage. Specific rotations were determined in ethanolic solutions (*c*, 0·5–1) at 15–20°. Al_2O_3 (Hopkin & Williams) was treated as previously described (Brooks & Norymberski, 1953). Microanalyses are by Weiler and Strauss, Oxford; samples for analyses were prepared by sublimation in a high vacuum, unless otherwise stated.

Oxidation of pregnanediol glucuronide with sodium bismuthate

Crude sodium pregnanediol glucuronidate of m.p. 255–260° was obtained from pregnancy urine essentially by the method of Venning (1937, 1938) (cf. Haslewood, 1950). This product (310 mg.) was dissolved in aqueous acetic acid (50%, v/v, 75 ml.) and shaken with $NaBiO_3$ (A.R., 7·5 g.) for 30 min. at room temperature; water (75 ml.) and ethyl acetate (150 ml.) were added and the shaking was continued for 10 min. The excess of reagent was filtered off with suction, the two layers of the filtrate were separated and the aqueous phase was extracted with ethyl acetate (4 × 100 ml.). The extract was washed successively with water (50 ml.), saturated $NaHCO_3$ solution (7 × 50 ml.), and water (3 × 50 ml.); it was dried with anhydrous Na_2SO_4 and evaporated to dryness. The crude product (pale-yellow gum, 175 mg.) was dissolved in benzene:ethyl acetate (99:1) and chromatographed on Al_2O_3 (5 g.) as shown in Table 1.

Fraction 1–3. 3α-*Formoxypregnan*-20-*one* (II*b*). Attempts to crystallize this fraction were unsuccessful and isolation of the pure formate (II*b*) was abandoned. The material was dissolved in a little light petroleum and put on a column of Al_2O_3 (1 g.). The next day the column was eluted successively with light petroleum (10 ml.), benzene (10 ml.) and benzene:ethyl acetate (90:10, 10 ml.). The first two eluates on evaporation yielded gummy residues. The last fraction gave crystals (12 mg.) which were recrystallized from light petroleum as needles (9 mg.), m.p. 143–146°, not depressed on admixture with authentic 3α-hydroxypregnan-20-one. $[\alpha]_D$ +106°. (Found: C, 79·7; H, 10·5. Calc. for $C_{21}H_{34}O_2$: C, 79·2; H, 10·8%.)

Fraction 4–8. 3α-*Formoxypregnan*-20α-*ol* (I*b*). This fraction was recrystallized twice from light petroleum to give thick prisms (15 mg.) of the 3α-*formoxy compound*, m.p. 114–116° (with change to needles at 106°); $[\alpha]_D$ +56°. (For analysis, a sample was dried in a high vacuum at 100°. Found: C, 75·6; H, 10·3. $C_{22}H_{36}O_3$ requires: C, 75·8; H, 10·4%.)

A sample (5·2 mg.) of the pure formate (I*b*) was hydrolysed on Al_2O_3 as described for 3α-formoxypregnan-20-one (II*b*). Benzene:ethanol (96:4) eluted pure pregnane-3α:20α-diol (4·9 mg.), m.p. 237–239°, undepressed by authentic material. $[\alpha]_D$ +25°. (Found: H, 11·5. Calc. for $C_{21}H_{36}O_2$: H, 11·3%. Owing to breakage of the CO_2 absorption tube no value for carbon is available.)

Table 1. *Chromatography of product from bismuthate oxidation of pregnanediol glucuronide*

Fraction no.	Eluent	Volume (ml.)	Eluate: Weight (mg.)	Eluate: M.p.
1–3	Benzene:ethyl acetate: (99:1)	18	25	—
4–8	(99:1)	30	33	80–107
9	(99:1)	6	6	—
10–14	(95:5)	30	12	131–147
15–22	(95:5 and 90:10)	50	5	90–140
23–25	(90:10)	18	1	185–195
26–38	(80:20 and 50:50)	80	33	200–235
39–41	Ethyl acetate	18	6	130–180
42–45	—	24	2	—
46–48	Ethyl acetate:ethanol: (98:2)	18	2	175–185
49–53	(98:2)	30	6	—
54–55	(96:4)	100	21	—
56–57	(80:20)	200	23	—

Oxidation of the formate I*b* (17·5 mg., obtained by evaporation of the mother liquors) with CrO_3 (3·75 mg., 1·1 molar equivalents) in glacial acetic acid (2 ml.) at room temperature yielded, after the usual working-up, a gummy neutral product (17·2 mg.) which failed to crystallize. It was hydrolysed on Al_2O_3 as described above; benzene:ethyl acetate (90:10) eluted a crystalline fraction (13 mg.) which, recrystallized from light petroleum, gave needles, m.p. 145–147°, identical (mixed m.p.) with authentic 3α-hydroxypregnan-20-one; $[\alpha]_D$ +108°. (Found: C, 78·8; H, 10·8. Calc. for $C_{21}H_{34}O_2$: C, 79·2; H, 10·8%.)

Fraction 10–14. *3α-Hydroxypregnan-20-one* (II*c*). Recrystallization of this fraction from light petroleum yielded needles (10 mg.), m.p. 144–146°, not depressed on admixture with authentic 3α-hydroxypregnan-20-one, $[\alpha]_D$ +105°. (Found: C, 78·5; H, 10·6. Calc. for $C_{21}H_{34}O_2$: C, 79·2; H, 10·8%.)

Fraction 26–38. *Pregnane-3α:20α-diol* (I*c*). This fraction was recrystallized from acetone to give plates (27 mg.), m.p. 236–237°, undepressed by authentic pregnane-3α:20α-diol, $[\alpha]_D$ +31°. (Found: C, 78·4; H, 11·1. Calc. for $C_{21}H_{36}O_2$: C, 78·7; H, 11·3%.)

Isolation of pregnane-3α:20α-diol from pregnancy urine

A 24 hr. urine specimen, obtained from a pregnant woman (38th week), was made up to 2·0 l. with distilled water. Three samples of 500 ml. were taken for the isolation of pregnane-3α:20α-diol by the following methods (*A*, *B* and *C*).

A. From urine treated with sodium bismuthate. Glacial acetic acid (500 ml.) and $NaBiO_3$ (A.R., 150 g.) were added to a sample (500 ml.) of the diluted urine and the mixture was shaken for 30 min. at room temperature. The excess of reagent was filtered off with suction and washed with methanol (50 ml.). A few ml. of a dilute $NaHSO_3$ solution were added to the filtrate to remove a slight suspension of bismuthate; the clear solution was diluted with distilled water (1·0 l.) and saturated with ethyl acetate (400 ml.). The mixture was extracted with ethyl acetate in a cascade extractor (Kies & Davis, 1951) consisting of four units, each unit containing 400 ml. of the solvent. The extract was washed with water (500 ml.) and then evaporated to dryness, yielding a dark, gummy residue (940 mg.). The crude extract was hydrolysed by boiling for 10 min. with 10% ethanolic (90%, v/v) NaOH (12 ml.); the mixture was diluted with water (100 ml.) and extracted with $CHCl_3$ (5 × 20 ml.). The partly crystalline, neutral product (55 mg.) was separated into three fractions by chromatography on Al_2O_3 (1·0 g.): benzene:ether (90:10, 60 ml.) eluted gummy material (21 mg.), benzene:ethanol (95:5, 30 ml.) gave yellowish crystals (15 mg.) and benzene:ethanol (90:10, 30 ml.) eluted traces of amorphous material. The second fraction was sublimed in a high vacuum at 190° to give a colourless sublimate (11 mg.) and a brown, gummy residue. The sublimate consisted of two fractions, one formed immediately above the heating block, the other about 2 cm. higher; the two fractions were separated by cutting the sublimation tube. The lower fraction yielded, on recrystallization from acetone, plates (5·3 mg.), m.p. 235–238°, undepressed by authentic pregnane-3α:20α-diol, $[\alpha]_D$ +30°. (Found: C, 78·5; H, 10·9. Calc. for $C_{21}H_{36}O_2$: C, 78·7; H, 11·3%.)

The upper sublimate was recrystallized from light petroleum and subsequently sublimed in a high vacuum at 80–90° to give feathery needles (1·2 mg.), m.p. 92–112°, $\lambda_{max.}$ 210 and 223 mμ., $E^{1\,cm.}_{1\%}$ 447 and 467. The product gave no colour with tetranitromethane nor with the Zimmermann reagent.

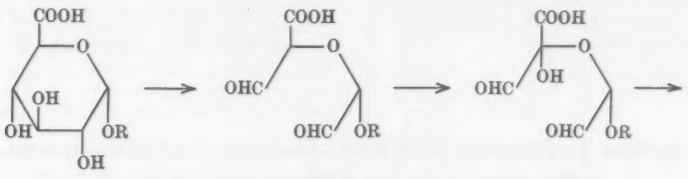
B. From acid-hydrolysed urine. The diluted urine sample (500 ml.) was brought to the boil, 10N-HCl (50 ml.) was added and boiling continued for 10 min. The mixture was cooled and glacial acetic acid (400 ml.), water (1·0 l.), sodium acetate (50 g.), methanol (50 ml.) and ethyl acetate (400 ml.) were added. Extraction, alkaline hydrolysis of the crude extract (480 mg.) and chromatography of the neutral product (30 mg., partly crystalline) were carried out as described under *A*. The first chromatographic fraction (12 mg.) consisted of a yellow gum; the second fraction (12 mg.) was crystalline and, on heating to 190° in a high vacuum, gave a colourless, partly oily sublimate (9·7 mg.); the volatile by-product described under *A* was not observed. The sublimate when crystallized from acetone gave plates (4·6 mg.) of pregnane-3α:20α-diol, m.p. 234–235°, $[\alpha_D]$ +30°. (Found: C, 78·8; H, 11·4. Calc. for $C_{21}H_{36}O_2$: C, 78·7, H, 11·3%.)

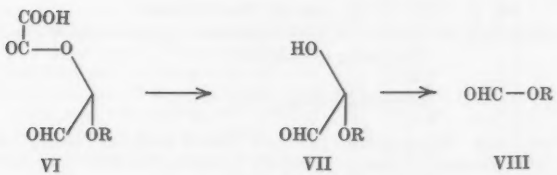
C. From untreated urine. Glacial acetic acid (500 ml.), water (1·0 l.), methanol (50 ml.) and ethyl acetate (400 ml.) were added to the diluted urine sample (500 ml.). The mixture was extracted, the crude extract (710 mg.) hydrolysed and the neutral product (14 mg. yellow gum) chromatographed as described under *A*. The first chromatographic fraction was a yellow gum (9 mg.); the second fraction was also gummy and gave only traces of an amorphous sublimate on heating in a high vacuum.

DISCUSSION

Oxidative fission of glycosides by sodium bismuthate has not been previously described; analogous reactions with periodate are, however, well known. Huebner, Lohmar, Dimler, Moore & Link (1945) have oxidized bornyl glucopyranosiduronic acid (III) with periodate and obtained bornyl formate (VIII) in 36% yield. The mechanism of this reaction has been postulated by Huebner *et al.* (1945) (see also Halsall, Hirst & Jones, 1947) as follows: glycol fission of bornyl glucopyranosiduronic acid (III) is followed by hydroxylation of the dialdehyde IV to give the hydroxyaldehyde V; V is oxidized to the oxalate VI which gives glyoxal hemiacetal (VII) on hydrolysis; VII is finally oxidized to bornyl formate (VIII). Since the hypothetical intermediates V and VII have hemiacetal structures, it is conceivable that hydrolysis occurs in competition with their oxidation, in which case glyoxal bornyl hemiacetal (VII) would give borneol as well as bornyl formate. The experimental evidence of Huebner *et al.* (1945) does not exclude this possibility. The above consideration applies also to the bismuthate oxidation of pregnanediol glucuronide: whatever the actual mechanism of this reaction, the isolation of the formates I*b* and II*b* is best explained by the oxidative cleavage of initially formed glyoxal hemiacetals; their hydrolysis would equally well explain the formation of the free alcohols I*c* and II*c*. However, the last two compounds may have been formed by hydrolysis of their formates during the working-up or during chromatography on alumina, though in both operations care was taken to avoid deformylation.

Whether oxidation of glucosiduronic acids with sodium bismuthate produces formates exclusively or whether alcohols are formed as well does not affect the application of the reaction to the cleavage of urinary glucosiduronic acids *in situ*, since either group of products will be extractable from urine. The quantitative recovery of pregnane-3α:20α-diol from bismuthate-treated urine suggests the use of this procedure as an alternative to the enzymic hydrolysis with β-glucuronidase. The concurrent degradation of corticosteroids by bismuthate, while imposing an obvious limitation on the applicability of the method, can be used to advantage as for instance in the differential estimation of glucosiduronic acids and sulphates of 'total 17-ketosteroids' (Norymberski, 1953).





R = bornyl, $C_{10}H_{17}$.

In the preceding paragraphs only the oxidative fission of glucopyranosiduronic acids has been considered, the pyranose form being the preferred structure of naturally occurring glucosiduronic acids. The degradation of a glucofuranosiduronic acid,

RO—CH—CHOH—CHOH—CH—CHOH—COOH,
|————O————|

may be expected to proceed differently with periodate and with bismuthate, since only the latter reagent can cleave α-hydroxyacids (Rigby, 1950).

SUMMARY

1. 'Pregnanediol glucuronide' from human pregnancy urine was oxidized with sodium bismuthate to give 3α-hydroxypregnan-20-one, pregnane-3α:20α-diol and their 3α-formates.

2. Pregnane-3α:20α-diol was extracted from pregnancy urine treated with sodium bismuthate in approximately the same yield as from acid-hydrolysed urine.

One of us (J.K.N.) was the holder of an Empire Rheumatism Council Research Fellowship during the period in which this work was done. We are much indebted to Prof. G. F. Marrian, F.R.S., for a sample of 3α-hydroxypregnan-20-one and to Dr L. P. de Abrew for making available to us specimens of pregnancy urine.

REFERENCES

Brooks, C. J. W. & Norymberski, J. K. (1952). *Chem. & Ind.* p. 804.
Brooks, C. J. W. & Norymberski, J. K. (1953). *Biochem. J.* **55**, 371.
Goldfine, M. M. & Cohen, S. L. (1953). *Endocrinology*, **52**, 597.
Halsall, T. G. Hirst, E. L. & Jones, J. K. N. (1947). *J. chem. Soc.*, p. 1427.
Haslewood, G. A. D. (1950). In *Hormone Assay*, ed. by Emmens, C. W., p. 459. New York: Academic Press.
Huebner, C. F., Lohmar, R., Dimler, R. J., Moore, S. & Link, K. P. (1945). *J. biol. Chem.* **159**, 503.
Kies, M. W. & Davies, P. L. (1951). *J. biol. Chem.* **189**, 637.
Koechlin, B. A., Kritchevsky, T. H. & Gallagher, T. F. (1950). *J. biol. Chem.* **184**, 393.
Levin, R. H., McIntosh, A. V., jun., Spero, G. B., Rayman, D. E. & Meinzer, E. M. (1948). *J. Amer. chem. Soc.* **70**, 511.
Norymberski, J. K. (1952). *Nature, Lond.*, **170**, 1074.
Norymberski, J. K. (1953). *Mem. Soc. Endocrinol.* **2** (in the Press).
Norymberski, J. K., Stubbs, R. D. & West, H. F. (1953). *Lancet*, **1**, 1276.
Rigby, W. (1950). *J. chem. Soc.*, p. 1907.
Steinegger, E. & Katz, A. (1947). *Pharm. Acta Helvet.* **22**, 1.
Sutherland, E. S. & Marrian, G. F. (1947). *Biochem. J.* **41**, 193.
Venning, E. H. (1937). *J. biol. Chem.* **119**, 473.
Venning, E. H. (1938). *J. biol. Chem.* **126**, 595.

Active Transport of Water, Sodium, Potassium and α-Oxoglutarate by Kidney-cortex Slices

By R. WHITTAM and R. E. DAVIES

Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield

(*Received* 1 *July* 1953)

Previous experiments from this Department (Terner, Eggleston & Krebs, 1950; Krebs, Eggleston & Terner, 1951) and by Mudge (1951*a*, *b*) and Aebi (1953) have shown that slices of some animal tissues suspended in a saline medium may lose or gain potassium ions according to the conditions. The present work with kidney-cortex slices is a study of the movements of sodium ions and α-oxoglutarate ions (added as substrate) and of water which accompany the movements of potassium. Part of this work has been communicated to the Biochemical Society (Whittam & Davies, 1953).

EXPERIMENTAL

Material and procedure. As it was necessary to measure the fresh weight of tissue slices before incubation, contact between tissue and medium during the slicing had to be avoided. Guinea-pigs were stunned and bled from the carotids. The kidneys were at once removed, cut longitudinally and the cortex was sliced with a dry razor according to the method of Deutsch (1936). Slices, 0·3–0·4 mm. thick, were spread on hardened filter paper (Whatman no. 541) and rapidly blotted to remove blood, urine and cell debris. The outer slices were usually rejected; samples (100–200 mg.) of the inner slices were then weighed on a torsion balance and put into 2·0 ml. of medium in 15 ml. stoppered conical cups. In anaerobic experiments, a stick of yellow phosphorus was placed in the centre well. The cups were shaken at 130 oscillations/min. (amplitude 2 cm.) at 37° in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Company, Chicago, U.S.A.), or at 0° in a mechanical rocker. After incubation the slices were removed, rapidly blotted and reweighed.

Media. Phosphate salines were used when media, gassed with O_2, were required at various pH values, which were checked with a glass electrode before and after incubation. They were similar to medium III of Krebs (1950*b*), without the addition of the organic substrates. In all other experiments, bicarbonate saline (Krebs & Henseleit, 1932) gassed with 5% CO_2 and either 95% O_2 or 95% N_2 at 37°, or 2·8% CO_2 and either 97·2% O_2 or 97·2% N_2 at 0° was used. The pH at both 37 and 0° was thus 7·4. The media used contained 6mM-K, 155–160mM-Na and usually 10mM α-oxoglutarate as substrate. A hypotonic (approx. 0·2 osM) medium was made by diluting 2 vol. of the medium with 1 vol. of water. (The osmolar concentration (osM) is obtained by adding together the molar concentrations of all the ions present, cf. Robinson, 1950.)

Water content of tissue. The tissue water was taken to be equal to the loss of weight on drying the tissue overnight at 105°.

Determination of α-oxoglutarate. The slices, or 1 ml. samples of the media, were each placed in 10 ml. centrifuge tubes containing 2·0 ml. of 10% (w/v) trichloroacetic acid (TCA). After breaking up the tissue with a glass rod the tubes were centrifuged. Samples of the supernatants were removed (0·1 ml. from the media, 1·0 ml. from the tissue extracts) and the volume made to 4·0 ml. with TCA. The tubes were then placed for 5 min. in a boiling-water bath to decompose any acetoacetic or oxaloacetic acids present (Markees, 1951; Lichtenbell & Florijn, 1952; Kogut & Podoski, 1953).

α-Oxoglutaric acid was estimated by the method of Goodwin & Williams (1952), which gave reproducible results only when freshly distilled diethyl ether was used for the extraction of the 2:4-dinitrophenylhydrazones.

Determination of sodium and potassium. Samples (0·4–0·5 ml.) of the TCA extracts of the tissue were diluted to 10·0 ml. either with 0·0382% (w/v) KCl (20 mg. K/100 ml.) or 0·84% (w/v) NaCl (330 mg. Na/100 ml.) for estimations of Na or K by flame photometry according to the method of Domingo & Klyne (1949) with the modifications of Terner *et al.* (1950). Samples of the TCA extracts of the medium (0·15 ml. for Na and 1·5 ml. for K) were also diluted to 10 ml. as above. In cases where the water content had been determined first, an amount of 16N-HNO_3 about equal to the wet weight of the tissue was added to the dried tissue and the mixture either left overnight at room temperature or kept for 30 min. at 100°. The acid digest was diluted to 15·0 ml. with water, and 5·0 ml. samples were used for analysis. For estimations of Na, 0·4 ml. of 0·955% (w/v) KCl and 4·6 ml. of water were added before flame photometry; for estimations of K, 0·5 ml. of 16·8% (w/v) NaCl and 4·5 ml. of water were added.

For estimations of Na in the medium or plasma, 0·05 ml. was diluted to 10·0 ml. with 0·0382% (w/v) KCl. For estimations of K in the medium or plasma, 0·5 ml. was diluted to 10·0 ml. with 0·84% (w/v) NaCl.

These procedures gave the same values as those of Terner *et al.* (1950) which were designed for the determination of only either Na or K in the samples. Neither the TCA nor the HNO_3 in the quantities used interfered with the estimations. Each reading was preceded and followed by a standard solution, and at least four readings were taken of each sample in all cases. The standard error of the mean values for each sample was about 1%.

RESULTS

Sodium and potassium contents of kidney cortex and plasma in vivo

Table 1 gives values for the sodium and potassium contents of kidney-cortex slices before incubation. Inner slices of the tissue were found to contain more sodium (10·2 ± 3·0 μmoles/g. tissue), and less potassium (13·0 ± 2·3 μmoles/g. tissue) than the first outer slices, although the difference of the sums of sodium and potassium contents was not significant (2·8 ± 3·9 μmoles/g. tissue). The outer slices, which were uncut on one side, and different from the rest of the cortex, were not used in the following experiments. The sodium and potassium concentrations in plasma were 144 ± 0·65(4) mM and 7·30 ± 0·089(4) mM, respectively.

Changes in the sodium, potassium and water contents of kidney-cortex slices on incubation in vitro

Aerobic incubation at 37°. About one-fifth of the tissue potassium was replaced by sodium after 40 min. incubation when no substrate was added (Table 2). When 10 mM α-oxoglutarate was present, the potassium content of the tissue was the same as the initial one; the sodium content was increased.

Table 1. *Sodium, potassium and water contents of guinea pig kidney-cortex slices before and after incubation* in vitro

(Slices of tissue (0·1–0·2 g.) incubated 30–50 min. in 2·0 ml. of bicarbonate saline, pH 7·4, containing 10 mM α-oxoglutarate, and gassed with 5% CO_2 + either 95% O_2 or 95% N_2 at 37°, or 2·8% CO_2 + either 97·2% O_2 or 97·2% N_2 at 0°. Mean values given ± S.E. with number of observations in parentheses. The concentration gradient is the ratio: concentration in tissue water/concentration in external medium. For other details see text.)

Material	Experimental conditions	Temp. of incubation (°)	Amounts of cations in tissue (μmoles/g. tissue) Na^+	K^+	$Na^+ + K^+$	Water content of tissue (%)	Excess amounts of $Na^+ + K^+$ in tissue relative to medium μmoles/g. tissue	μmoles/g. water	Concentration gradient of $Na^+ + K^+$
Outer slices	Before incubation	—	53·2 ± 2·3 (15)	89·4 ± 2·1 (17)	142·6 ± 3·3 (15)	76·9 ± 0·6 (4)	32*	25*	1·21 ± 0·01*
	Before incubation	—	63·4 ± 1·9 (14)	76·4 ± 0·9 (19)	139·8 ± 2·1 (14)	77·7 ± 0·2 (19)	27*	21*	1·18 ± 0·01*
Inner slices	After incubation: Aerobic	37	90·2 ± 1·8 (24)	75·3 ± 1·6 (24)	165·5 ± 2·4 (24)	79·7 ± 0·3 (24)	46	37	1·29 ± 0·03
	After incubation: Aerobic	0	112 ± 2·9 (13)	30·9 ± 0·7 (13)	142·9 ± 3·0 (13)	83·0 ± 0·5 (7)	11	9·2	1·07 ± 0·04
	After incubation: Anaerobic	37	114 ± 2·2 (9)	27·5 ± 1·0 (9)	141·5 ± 2·5 (9)	82·7 ± 0·3 (4)	10	9·1	1·06 ± 0·03
	After incubation: Anaerobic	0	109 ± 2·9 (7)	32·2 ± 1·7 (7)	141·2 ± 3·3 (7)	83·5 (2)	8	9·0	1·05 ± 0·04

* These values are relative to plasma.

Table 2. *Changes in the sodium and potassium contents of guinea pig kidney-cortex slices on incubation* in vitro

(Slices of tissue (0·1–0·2 g.) incubated in 2·0 ml. of bicarbonate saline, pH 7·4; gas phase, 5% CO_2 + either 95% O_2 or 95% N_2 at 37°.)

Expt. no.	Experimental conditions	Concentration of α-oxo-glutarate in medium before incubation (mM)	Concentration of 2:4-dinitro-phenol in medium before incubation (mM)	Period of incubation (min.)	Amounts of cations in tissue (μmoles/g. tissue) Before incubation Na^+	K^+	$Na^+ + K^+$	After incubation Na^+	K^+	$Na^+ + K^+$
1	Aerobic	0	0	40	56·0	78·8	134·8	74·5	63·3	137·8
		2	0	40	56·0	78·8	134·8	76·3	65·8	142·1
		4	0	40	56·0	78·8	134·8	75·4	73·7	149·1
		6	0	40	56·0	78·8	134·8	75·6	78·3	153·9
		8	0	40	56·0	78·8	134·8	74·3	78·5	152·8
		10	0	40	56·0	78·8	134·8	84·8	79·5	164·3
		10*	0	40	56·0	78·8	134·8	78·9	81·8	160·7
2	Aerobic	10	0	35	62·6	75·7	138·3	91·8	71·3	163·1
		10	0	50	62·6	75·7	138·3	87·6	71·8	159·4
		10	0·2	35	62·6	75·7	138·3	110	39·9	149·9
		10	0·2	50	62·6	75·7	138·3	112	35·4	147·4
		10	0·2†	50	62·6	75·7	138·3	107	42·6	149·6
3	Aerobic	10	0	40	61·4	77·9	139·3	91·8	71·3	163·1
		10	0	50	61·4	77·9	139·3	87·6	71·8	159·4
		10	0	90	61·4	77·9	139·3	88·9	56·6	147·5
		10	0	120	61·4	77·9	139·3	98·5	56·2	154·7
		10	0	240	61·4	77·9	139·3	130	27·2	157·2
		10	0	360	61·4	77·9	139·3	134	23·8	157·8
4	Anaerobic	10	0	2	68·2	76·1	144·3	95·8	47·1	142·9
		10	0	5	68·2	76·1	144·3	104	39·8	143·8
		10	0	16	68·2	76·1	144·3	116	29·2	145·2
		10	0	34	68·2	76·1	144·3	120	29·8	149·8
		10	0	120	68·2	76·1	144·3	131	14·5	145·5
		10	0	240	68·2	76·1	144·3	145	11·2	156·2
		10	0	360	68·2	76·1	144·3	141	9·8	150·8

* The saline also contained 20 mM glucose. † 2:4-Dinitrophenol added after 35 min. incubation.

Table 3. *Effect of* pH *on sodium, potassium and water contents of guinea pig kidney-cortex slices on incubation* in vitro

(Slices of tissue (0·1–0·2 g.) were washed in three 10 ml. portions of phosphate saline of appropriate pH and then incubated for 40 min. at 37° in 4·0 ml. of phosphate saline containing 10 mM α-oxoglutarate; gas phase O_2; 0·2 ml. of 2N-NaOH in centre well. The concentration gradient is the ratio: concentration in tissue water/concentration in external medium.)

Experimental conditions	pH of medium during incubation	Amounts of cations in tissue (μmoles/g. tissue) Na^+	K^+	$Na^+ + K^+$	Water content of tissue (%)	Concentrations of cations in tissue water (μmoles/g. tissue water) Na^+	K^+	$Na^+ + K^+$	Concentration gradient of cations Na^+	K^+	$Na^+ + K^+$
Slices before incubation		55·7	76·7	132·4	77·9	71·5	98·5	170·0	0·50*	13·5*	1·13*
Slices after incubation	3·6	112	9·1	121·1	84·1	133	10·8	143·8	0·86	1·35	0·88
	4·6	93·8	45·1	138·9	80·6	116	55·9	171·9	0·75	6·8	1·06
	5·7	83·5	58·7	142·2	79·5	105	73·8	178·8	0·67	10·0	1·10
	5·9	73·0	66·7	139·7	78·1	93·5	85·3	178·8	0·60	14·0	1·10
	6·9	77·7	83·8	161·5	80·9	95·0	104	199·0	0·61	18·2	1·23
	7·5	93·5	72·2	165·7	80·6	116	89·5	205·5	0·74	15·7	1·27
	7·6	88·6	71·2	159·8	79·7	111	89·2	200·2	0·71	15·7	1·24
	7·8	79·2	76·1	155·3	79·9	99·1	95·3	194·4	0·64	16·7	1·20
	8·3	98·0	47·7	145·7	82·3	119	58·0	177·0	0·76	7·0	1·09
	9·4	117	23·8	140·8	84·0	139	28·3	167·3	0·89	3·3	1·03

* These values are relative to plasma.

Glucose had no additional effect. After incubation in media with lower concentrations of α-oxoglutarate, the slices contained less potassium and more sodium than initially (Table 2).

The time curves of the sodium and potassium contents during incubation with 10 mM α-oxoglutarate are shown in Fig. 1. There was an initial gain of sodium and loss of potassium during the first 5 min., but this was largely reversed after 20–30 min. incubation, after which a steady state was maintained for at least a further 20 min. The sodium, potassium and water contents in this steady state are also shown in Table 1. They represent changes from the initial state of $+26{\cdot}8 \pm 2{\cdot}6$ μmoles/g. tissue for sodium and $-1{\cdot}1 \pm 1{\cdot}9$ μmoles/g. tissue for potassium and $+2{\cdot}0 \pm 0{\cdot}4$ for the percentage water content. The increase in the sum of sodium and potassium contents was thus $+25{\cdot}7 \pm 3{\cdot}2$ μmoles/g. tissue and this was all due to a net uptake of sodium in excess of that required to balance the potassium changes. After incubations of more than 50 min. the sodium and potassium contents of the tissue progressively increased and decreased, respectively (Table 2).

Table 3 gives the sodium, potassium and water contents of tissue after 40 min. incubation in phosphate media of pH values between 3·6 and 9·4. The optimum pH for maintaining the approximate physiological concentrations of sodium, potassium and water was not critical and about pH 7. At this pH the potassium concentration of the tissue water was about 1600 % greater than that of the medium and the sodium concentration was about 40 % less than that of the medium. The sum of sodium and potassium concentrations in the tissue water was 23 % greater than that of the medium. This was similar to the effect observed after 40 min. incubation in physiological bicarbonate saline when the comparable figure was 29 % (Table 1); in both cases there was thus an increase in the sum of sodium and potassium concentrations of the tissue water.

At higher or lower pH values than 7 the concentration differences of sodium and potassium between the tissue water and medium became progressively smaller but did not completely disappear. Even at pH 3·6, there was no complete levelling out, the potassium concentration of the tissue water being 35 % greater than that of the medium and the sodium concentration 14 % less than that of the medium.

In contrast to the uneven distributions of the individual ions between the tissue water and medium, the sum of the sodium and potassium concentrations became progressively more constant at pH values increasingly higher or lower than 7. At pH 9·4 the sum of sodium and potassium concentrations in the tissue water and medium was approximately the same. At pH 3·6, however, the sum of the sodium and potassium concentrations of the tissue water was 12 % less than that of the medium. This may be a result of the decrease in the number of anions of high molecular weight in the tissue.

2:4-Dinitrophenol caused the tissue to gain sodium and lose potassium when it was added after the slices had reached the steady state after 35 min. incubation (Table 2). When added at the start of the incubation it did not affect the initial leakages but prevented both the subsequent extrusion of sodium and the accumulation of potassium by the tissue.

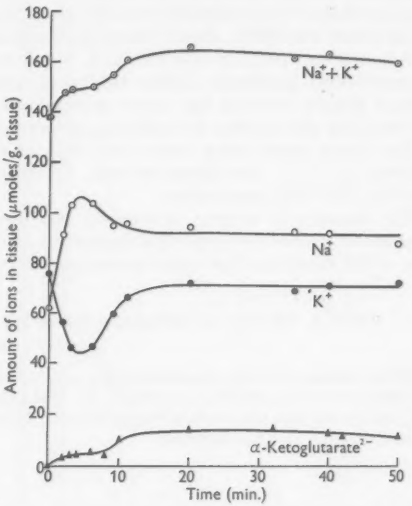


Fig. 1. Changes in the sodium, potassium and α-oxoglutarate contents of guinea pig kidney-cortex slices during incubation *in vitro*. Slices of tissue (0·1–0·2 g.) incubated in 2·0 ml. bicarbonate saline, pH 7·4, containing 10 mM α-oxoglutarate and gassed with 5 % CO_2 + 95 % O_2, temp. 37°.

Aerobic incubation at 0°. As at 37°, potassium was lost and sodium taken up at 0°, but at this temperature the tissue did not reverse these changes nor was there an increase in the sum of sodium and potassium contents. After from 30 to 50 min. incubation the potassium content was 40 % of the initial value (Table 1); after 6 hr. it was 26 % whilst slices frozen and thawed before incubation contained only 17 % of the initial amount (Table 4). Since the sum of the sodium and potassium contents remained the same as initially, the changes in the sodium content were equal and opposite to those of potassium. Thus the sodium content increased by 77 % after from 30 to 50 min. incubation and by 112 % after 6 hr. incubation, whilst previously frozen and thawed tissue contained 120 % more sodium than the initial tissue after 6 hr. incubation (Tables 1 and 4).

The ratio of the sum of sodium and potassium concentrations in tissue water/the sum of sodium and potassium concentrations in medium, which was 1·29 after incubation at 37°, was 1·07 after from 30 to 50 min. incubation at 0° and 1·05 after 6 hr. incubation at this temperature, whilst slices frozen and thawed before incubation at 0° gave a ratio of 0·99 (Table 4). Whilst the sum of the concentrations of sodium and potassium became the same in the tissue water and medium, the concentrations of the individual cations did not become equal. After 6 hr. incubation the potassium concentration of the tissue water was 160 % above that of the medium, and it was still 40 % greater for slices frozen and thawed before incubation (Table 4). The distribution of sodium between the tissue water and the medium was also uneven, the sodium concentration of the tissue water being lower than that of the medium by 13 % after about 40 min. incubation and 7 % after 6 hr. incubation.

The changes in sodium, potassium and water contents at 0° were reversed by subsequent incubation at 37° (Table 5). The initial potassium content was completely restored. The sodium content fell but did not reach the original level, probably because of an uptake of α-oxoglutarate at 37° which, as will be shown later, may also account for the rise in the sum of sodium and potassium contents.

Anaerobic incubation. When the tissue was incubated under anaerobic conditions there was a rapid initial gain of sodium and loss of potassium similar to that under aerobic conditions, but whilst aerobically at 37° these changes were reversed on prolonged incubation, they became progressively greater anaerobically. The rate of change in sodium and potassium contents became slow after the first 30 min. incubation, whilst the sum of sodium and potassium contents remained about the same as that of the initial tissue after various periods of incubation (Table 2).

The tissue contained about 40 % of the initial potassium after from 30 to 50 min. incubation at either 37° or 0°. After 6 hr. incubation more potassium was lost at 37° than at 0°, the amounts then present being 13 and 23 % of the initial value, respectively. In all cases the change in sodium

Table 4. *Changes in the sodium, potassium and water contents of guinea pig kidney-cortex slices on incubation at* 0°

(Slices of tissue (0·1–0·2 g.) incubated for 6 hr. in 2·0 ml. of bicarbonate saline, pH 7·4, containing 10 mM α-oxoglutarate; gas phase, 2·8 % CO_2 + 97·2 % O_2 or 97·2 % N_2. Some slices, referred to as 'frozen' tissue, were alternately frozen on solid CO_2 and thawed four times before being incubated. The concentration gradient is the ratio: concentration in tissue water/concentration in external medium.)

Material	Treatment of tissue	Amounts of cations in tissue (μmoles/g. tissue) Na^+	K^+	$Na^+ + K^+$	Water content of tissue (%)	Concentration gradient Na^+	K^+	$Na^+ + K^+$
Slices before incubation		55·1	76·7	131·8	77·9	0·49*	13·3*	1·11*
Slices after incubation	Untreated, aerobic	117	20·3	137·3	84·8	0·93	2·6	1·05
	'Frozen', aerobic	121	13·0	134·0	87·4	0·95	1·4	0·99
	Untreated, anaerobic	111	17·8	128·8	85·3	0·89	2·4	0·98
	'Frozen', anaerobic	121	12·3	133·3	87·8	0·94	1·3	1·00

* These values are relative to plasma.

Table 5. *Reversibility of the changes in sodium, potassium and water contents of guinea pig kidney-cortex slices during incubation* in vitro

(Slices of tissue (0·1–0·2 g.) incubated in 2·0 ml. of bicarbonate saline, pH 7·4, containing 10 mM α-oxoglutarate, gassed with 5 % CO_2 + either 95 % O_2 or 95 % N_2 at 37°, or 2·8 % CO_2 + either 97·2 % O_2 or 97·2 % N_2 at 0°.)

Expt. no.	Material	Experimental conditions	Amounts of cations in tissue (μmoles/g. tissue) Na^+	K^+	$Na^+ + K^+$	Water content of tissue (%)
1	Slices before incubation	—	62·8	74·9	137·7	79·1
	Slices after incubation	27 min. at 0° (aerobic)	109	32·6	141·6	84·5
		28 min. at 0° then 54 min. at 37° (aerobic)	99	73·6	172·6	80·1
2	Slices before incubation	—	57·3	77·3	134·6	76·8
	Slices after incubation	34 min. anaerobic at 37°	120	29·8	149·8	82·0
		15 min. anaerobic then 32 min. aerobic at 37°	83·4	83·0	166·4	77·2

content was approximately equal and in an opposite direction to the change in potassium content (Tables 1, 2 and 4).

The ratio of the concentration of sodium or potassium in tissue water/the concentration of sodium or potassium in the medium, changed towards unity but did not reach it at either 37° or 0° with increasing periods of incubation. The nearest approach to unity was after 6 hr. incubation at 37°, when the potassium of the tissue water was only 20 % more than that of the medium; at 0° the comparable value was 140 %. Thus, in anaerobic conditions there was a more uniform distribution of sodium and potassium between the tissue water and medium at 37° than there was at 0°. Hence, as was also the case with tissue incubated aerobically at 0°, the sum of the concentrations of sodium and potassium in the tissue water approached that of the medium, whilst the separate distributions of sodium and potassium remained uneven.

Table 5 shows that the swelling of tissue and the changes in sodium and potassium contents during anaerobic incubation at 37° were reversed when the conditions were made aerobic.

Incubation in hypotonic medium. Swelling of kidney slices on incubation in saline medium (40 min.; 37°), as might be expected, was greater in hypotonic medium (0·2 osM) than isotonic medium (0·3 osM), the increases in water content from the initial state being 3·6 and 2·0 %, respectively. Despite this difference in swelling, the excess in the sum of sodium and potassium contents of the tissue relative to the medium was about 37 μmoles/g. tissue in each case. The sums of the concentrations of sodium and potassium in the tissue water were 38 and 29 % greater than those of the medium after incubation in 0·2 and 0·3 osM media, respectively, and these values are similar to those reported by Robinson (1952*a*) for the ratio of 'fixed base' between tissue water and suspending medium.

To find the rates of change of sodium, potassium and water contents, slices were pre-incubated under aerobic conditions at 37° for 35 min. in an isotonic medium, in order to allow the completion of the recovery processes, and then transferred to the hypotonic medium at 0°. The subsequent changes in sodium, potassium and water contents at 0° are shown in Fig. 2. The swelling of the tissue and the decrease in the sum of sodium and potassium contents were concomitant and complete within about 10 min., although slow changes in individual sodium and potassium contents still occurred even after 2 hr. There was a very rapid extrusion of sodium against a gradient before the subsequent uptake.

The sum of sodium and potassium concentrations of the tissue water at 0° in 0·2 osM medium was 23 % greater than that of the medium, whereas in isotonic medium (0·3 osM) the comparable figure was 7 %. Thus, despite the fall in the sum of sodium and potassium contents of the tissue on transfer to the hypotonic medium, the excess of these ions over the content in the medium was 22 μmoles/g. tissue; in isotonic medium this excess was only 9·2 μmoles/g. tissue (Table 1). This difference would not be expected if the excess of cations were unexchangeably bound in the tissue.

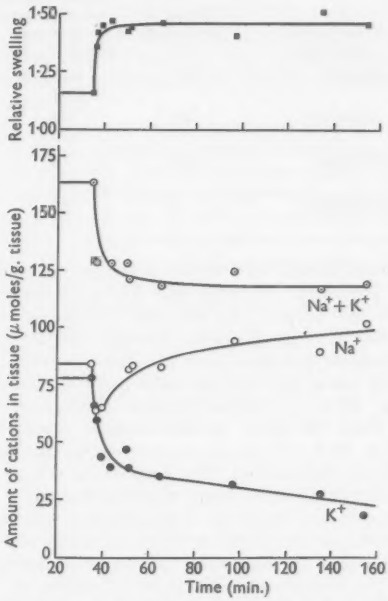


Fig. 2. Changes in sodium and potassium contents and relative swelling of guinea pig kidney-cortex slices on incubation at 0°. Slices of tissue (0·1–0·2 g.) incubated in 2·0 ml. of isotonic (0·3 osM) bicarbonate saline, pH 7·4, at 37° for 35 min. and then transferred to 2·0 ml. of hypotonic (0·2 osM) bicarbonate saline, pH 7·4, at 0°. Relative swelling is $(100 - W)/(100 - W^1)$, where W and W^1 are water contents of the slices before and after incubation respectively.

Transport and metabolism of α-oxoglutarate

The α-oxoglutarate content of fresh guinea pig kidney cortex was 0·12 μmole/g. tissue (cf. Krebs, 1950*a*; LePage, 1950; Frohman, Orten & Smith, 1951) and was unaltered when the tissue was sliced. Table 6 and Fig. 1 show the amounts of α-oxoglutarate accumulated and metabolized by incubated tissue slices. During aerobic incubation at 37° for 40 min., the concentration of α-oxoglutarate in the tissue water reached a level 2·6 times greater than that of the medium. The quantity of α-oxoglutarate accumulated by the tissue during this time was 12·0 ± 0·6 μmoles/g. tissue. Under the

Table 6. *Accumulation and metabolism of α-oxoglutarate in guinea pig kidney-cortex slices during incubation* in vitro

(Slices of tissue (0·1–0·2 g.) incubated 40 min. in 2·0 ml. of bicarbonate saline, pH 7·4, containing 10 mM α-oxoglutarate; gas phase, 5% CO_2 + either 95% O_2 or 95% N_2 at 37°, or 2·8% CO_2 + either 97·2% O_2 or 97·2% N_2 at 0°. Determinations of α-oxoglutarate were made on incubated tissues and on media before and after incubation. The concentration gradient is the ratio: concentration in tissue water/concentration in external medium. Mean values are given ± S.E. The figures in parentheses represent the number of observations.)

Experimental conditions	Temp. of incubation (°)	Accumulation of α-oxoglutarate in tissue (μmoles/g. tissue)	Amount of α-oxoglutarate in medium after incubation (μmoles/ml.)	$Q_{\alpha\text{-oxoglutarate}}$ (μl./mg. dry wt./hr.)	Concentration gradient of α-oxoglutarate
Aerobic	37	12·0 ± 0·6 (13)	5·8 ± 0·4 (13)	−8·9 ± 0·9 (15)	2·6 ± 0·2
Anaerobic	37	3·1 (3)	7·6 (3)	−4·7 (3)	0·49
Aerobic	0	2·3 (3)	8·4 (3)	−3·1 (2)	0·33
Anaerobic	0	2·1 (2)	9·1 (2)	−1·1 (2)	0·28

same conditions, the increase in the sum of sodium and potassium contents was 25·7 ± 3·2 μmoles/g. tissue (see earlier). So the ratio of this increase/increase in α-oxoglutarate content was 2·1 ± 0·3. This means that equivalent net quantities of monovalent cation and divalent anion had been accumulated. However, this net uptake of anion must be less than the gross uptake, because during this period 53·3 ± 5·5 μmoles α-oxoglutarate/g. tissue had been metabolized. This is more than 4 times the amount found in the tissue after incubation.

In contrast, much less α-oxoglutarate was found in the tissue than in the medium and much less was metabolized during aerobic or anaerobic incubation at 0°, or anaerobic incubation at 37° (Table 6).

DISCUSSION

Electrochemical equivalent of potassium movements. The experiments of Terner *et al.* (1950) and Krebs *et al.* (1951) have shown that brain- and kidney-cortex slices lose potassium but can largely recover it when the suspending medium contains L-glutamate and glucose, or α-oxoglutarate. These findings raise the question of the electrochemically equivalent change which must accompany the movement of potassium to maintain electroneutrality. The present data indicate that potassium ions are exchanged for sodium ions: the movement of sodium is accompanied by a movement of sodium in the opposite direction (see also Mudge, 1951*a*, *b*, and Aebi, 1953).

Effect of energy supply. Conditions which either reduce energy production (anoxia, cooling) or energy utilization (addition of 2:4-dinitrophenol) prevent the transport of potassium and sodium in the recovery period. These movements, which are against concentration gradients and in opposite directions, thus represent an active transport. Unlike red blood cells, kidney slices cannot obtain sufficient energy for active transport from anaerobic glycolysis, as might be expected from the relatively low rate of glycolysis in kidney cortex. Unlike brain, kidney does not require glucose for optimum energy production, which is again expected, because kidney contains much greater quantities of oxidizable substrates than brain.

Concentration differences not dependent on energy supply. There remains an uneven distribution of sodium and potassium between tissue water and medium, even in the virtual absence of energy supplies, namely at 0° or anaerobically. This indicates that concentration gradients of some sodium and potassium ions can be maintained by mechanisms not requiring the expenditure of energy. This applies to about 12% of the tissue sodium and 4% of the tissue potassium. The phenomenon may be due to a Donnan effect or, what is not fundamentally different, to properties of cell constituents similar to those of ion-exchange resins. A simple Donnan effect would require that the ratios of the sodium concentration in tissue water/the sodium concentration in medium, and the potassium concentration in tissue water/the potassium concentration in medium, are the same. In fact, that for potassium is greater. However, such effects are also known in the case of some exchange resins and are attributed to preferential binding of potassium due to the smaller radius of the hydrated ion (Boyd, Schubert & Adamson, 1947).

Transport of α-oxoglutarate. The movement of sodium cannot all be explained as a counterbalance of potassium movement because more sodium than required can be taken up by the tissue under aerobic conditions. The surplus sodium can be accounted for as the cationic equivalent of α-oxoglutarate which

is accumulated concomitantly. An accumulation of α-oxoglutarate in the tissue cannot be explained by a Donnan effect. Being an anion, the α-oxoglutarate would be expected to be in lower concentration inside than outside the tissue, whilst the reverse holds. As the concentration gradients were only found in respiring tissue at 37°, the accumulation of α-oxoglutarate must also represent a case of active transport.

Intracellular osmotic pressure. Earlier workers have generally assumed that the intracellular fluid is isotonic with extracellular fluid (see Peters, 1944), but the work of Stern, Eggleston, Hems & Krebs (1949) and of Robinson (1950, 1952*b*) who showed that animal tissues swell when the energy supply is cut off, and of Opie (1949) who showed that this swelling can be prevented by placing tissues in hypertonic solutions, suggests that tissues are in fact hypertonic and have to pump out water continuously to prevent swelling. This interpretation has not been accepted by Conway & McCormack (1953) who found the freezing-point depression of fresh guinea pig tissues to be the same as that of plasma. A high intracellular osmotic pressure is also suggested by the fact that the sum of the concentrations of sodium and potassium in the tissue water can be much greater than in plasma (Conway & Hingerty, 1946; Davies, Davies, Francis & Whittam, 1952; Aebi, 1953). Since some of the tissue water is extracellular with concentrations of sodium and potassium similar to those in plasma, this sum must be even greater in the cell water. It is, however, not known to what extent cations are osmotically inactive within the cell. It is feasible that they are partly rendered inactive by immobile polyvalent anions (Kern, 1938), but it is unlikely that such anions play a major role in the distribution of electrolytes because they could hardly account for the characteristic behaviour of the tissue water and electrolytes, such as the changes which occur on addition of inhibitors, lowering of temperature and cessation of respiration.

Further work is therefore needed to measure the osmotic pressure in isolated tissue slices under a variety of conditions to see whether the changes in water content and ionic gradients are in fact compatible with a constant osmotic pressure across the cell wall, in agreement with the conclusion of Conway & McCormack (1953), or signify a difference in the osmotic pressure across the cell wall, as postulated by Robinson (1950).

SUMMARY

1. In extending previous work (Terner *et al.* 1950; Mudge, 1951*a*, *b*) it was found that slices of guinea pig kidney cortex lost 40 % of their initial potassium content to, and gained an extra 60 % sodium from, a saline medium during the first 5 min. of aerobic incubation at 37° and pH 7·4. These changes were reversed after 30 min. with 10 mM α-oxoglutarate as substrate.

2. During aerobic incubation at 37° for 40 min. the sum of sodium and potassium contents of the tissue rose by $25{\cdot}7 \pm 3{\cdot}2$ μmoles/g. tissue, whilst the content of dibasic α-oxoglutarate rose by the equivalent amount of $12{\cdot}0 \pm 0{\cdot}6$ μmoles/g. tissue. The sum of sodium and potassium concentrations in the tissue water was 1·29 times greater than that in the medium; the α-oxoglutarate concentration was 2·6 times greater. During this time $53{\cdot}3 \pm 5{\cdot}5$ μmoles α-oxoglutarate/g. tissue were metabolized.

3. All concentration gradients of sodium, potassium and α-oxoglutarate between slices and medium were reduced or disappeared during aerobic or anaerobic incubation at 0°, or anaerobic incubation at 37°.

4. The dry weight of the tissue decreased during incubation by 9·0 % at 37° in O_2 and by from 22 to 26 % at 37° in N_2 or at 0° in either O_2 or N_2.

5. During incubation of tissue in a hypotonic medium at 0°, the water content increased and the sum of sodium and potassium contents fell concomitantly. Movements of sodium and potassium separately continued after the movement of water was complete.

6. These results confirm that in guinea pig kidney-cortex slices the prevention of swelling, and the production and maintenance of sodium and potassium concentration gradients are dependent upon a supply of energy from respiration.

We wish to thank Prof. H. A. Krebs, F.R.S., for his interest and encouragement, and one of us (R.W.) wishes to acknowledge the receipt of a grant from the Agricultural Research Council. This work was aided by a grant from the Rockefeller Foundation.

REFERENCES

Aebi, H. (1953). *Helv. physiol. acta*, **11**, 96.
Boyd, G. E., Schubert, J. & Adamson, A. W. (1947). *J. Amer. chem. Soc.* **69**, 2818.
Conway, E. J. & Hingerty, D. (1946). *Biochem. J.* **40**, 561.
Conway, E. J. & McCormack, J. I. (1953). *J. Physiol.* **120**, 1.
Davies, F., Davies, R. E., Francis, E. T. B. & Whittam, R. (1952). *J. Physiol.* **118**, 276.
Deutsch, W. (1936). *J. Physiol.* **87**, 56*P*.
Domingo, W. R. & Klyne, W. (1949). *Biochem. J.* **45**, 400.
Frohman, C. E., Orten, J. M. & Smith, A. H. (1951). *J. biol. Chem.* **193**, 277.
Goodwin, T. W. & Williams, G. R. (1952). *Biochem. J.* **51**, 708.
Kern, W. (1938). *Z. phys. Chem.* (A), **181**, 249.
Kogut, M. & Podoski, E. P. (1953). *Biochem. J.* **55**, 800.
Krebs, H. A. (1950*a*). *Biochem. J.* **47**, 605.
Krebs, H. A. (1950*b*). *Biochim. biophys. Acta*, **4**, 249.
Krebs, H. A., Eggleston, L. V. & Terner, C. (1951). *Biochem. J.* **48**, 530.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
LePage, G. A. (1950). *Cancer Res.* **10**, 393.
Lichtenbell, H. V. M. & Florijn, E. (1952). *Biochim. biophys. Acta*, **8**, 349.
Markees, S. (1951). *Experientia*, **7**, 314.
Mudge, G. H. (1951*a*). *Amer. J. Physiol.* **165**, 113.
Mudge, G. H. (1951*b*). *Amer. J. Physiol.* **167**, 206.
Opie, E. L. (1949). *J. exp. Med.* **89**, 185.
Peters, J. P. (1944). *Physiol. Rev.* **24**, 491.
Robinson, J. R. (1950). *Proc. Roy. Soc.* B, **137**, 378.
Robinson, J. R. (1952*a*). *Nature, Lond.*, **169**, 713.
Robinson, J. R. (1952*b*). *Proc. Roy. Soc.* B, **140**, 135.
Stern, J. R., Eggleston, L. V., Hems, R. & Krebs, H. A. (1949). *Biochem. J.* **44**, 410.
Terner, C., Eggleston, L. V. & Krebs, H. A. (1950). *Biochem. J.* **47**, 139.
Whittam, R. & Davies, R. E. (1953). *Biochem. J.* **54**, vii.

The Enzymic Hydrolysis of Acid Anhydrides

By F. BERGMANN, M. WURZEL and E. SHIMONI
Department of Pharmacology, The Hebrew University, Hadassah Medical School, Jerusalem, Israel

(*Received* 21 *February* 1953)

According to recent investigations (Burgen, 1949; Wilson & Bergmann, 1950) tetraethyl pyrophosphate (TEPP) and related inhibitors of esterases are enzymically split into a phosphonium ion, which combines with the nucleophilic group in the active surface, and a negative ion, e.g. the diethyl phosphate ion. The combination 'enzyme-$P(OR)_2$' is

$$\text{enzyme-P(OR)}_2 \quad (\text{O above P})$$

thus analogous to the complex 'enzyme-C—R',

$$\text{enzyme-C(=O)—R}$$

which we assumed as intermediate in the hydrolysis of esters (Bergmann, Wilson & Nachmansohn, 1950). The main difference between the two combinations is that the enzyme-acyl complex is spontaneously split by the solvent, regenerating the active form of the enzyme, whereas the enzyme-phosphonium compound is stable, at least for the duration of a laboratory experiment.

The superiority of TEPP over trialkyl phosphates in the inhibition of esterases (Aldridge & Davison, 1952) shows that the anhydride linkage is split faster by esterases than the ester group in derivatives of phosphoric acid. It should therefore be expected that anhydrides of carboxylic acids will also be easily attacked by these enzymes. The experiments described in the present investigation substantiate this prediction.

MATERIALS AND METHODS

Substrates and inhibitors. The fatty acid anhydrides were commercial samples and were purified by fractionation *in vacuo*. Dibenzyl acetyl phosphate was prepared essentially as described by Bentley (1948), but the reaction with keten was carried out in the absence of solvent, the liquid end product serving as solvent for dibenzyl hydrogen phosphate. Tetrabenzyl pyrophosphate was synthesized by the method of Kenner, Todd & Weymouth (1952).

Enzyme preparations. Liver esterase was prepared from dog's liver by ammonium sulphate fractionation, as described earlier (Bergmann & Shimoni, 1952). The yellow solution, obtained after two subfractionations, contained 16·6 mg. protein/ml., as determined by precipitation with 20% (w/v) trichloroacetic acid. When diluted 1:100 and with diacetin 0·43M as substrate, this enzyme liberated 5·3 μmoles CO_2/ml./hr.

Acetylcholine esterase was prepared from the electric organ of *Torpedo marmorata*. The final preparation, when diluted 1:40 000 and with acetylcholine $3{\cdot}3 \times 10^{-3}$M as substrate, hydrolysed 6 μmoles/ml./hr. For some of our experiments the enzyme from *Electrophorus electricus* was used, which in a dilution of 1:1200 hydrolysed 6·6 μmoles acetylcholine/ml./hr.

Manometric methods. The buffer used as medium for these experiments contained 0·1M-NaCl, 0·026M-$NaHCO_3$ and 0·04M-$MgCl_2$. The pH was adjusted to 7·3. The gas phase consisted of 95% air and 5% CO_2. In the experiments with toluene as the organic phase, the solution of anhydride in this solvent (0·1–0·3 ml.) was placed in the side bulb of a

Warburg vessel and tipped into the main compartment at zero time. For a rapid attainment of constant manometer readings before mixing, it was found advantageous to add a small quantity (0·02 ml.) of toluene to the main vessel. The anhydrides were finally used in the following dilutions: acetic 1:100, propionic 1:100 and butyric 1:10. The relatively high concentration of the last compound was possible because butyric anhydride undergoes only slow spontaneous hydrolysis when its solution in toluene is brought into contact with bicarbonate buffer. In every case care was taken that the amount of buffer was in excess of that required to neutralize the total amount of acid to be expected after complete hydrolysis of substrate, and the pH of the mixtures was checked at the end of the experiments.

A certain difficulty in quantitative evaluation arises from the fact that the anhydride concentration in the organic phase decreases faster in the presence of enzyme than in the control, i.e. the absolute concentrations, which are equilibrated between the two layers, are equal only at zero time. This, however, makes the positive results obtained in our experiments the more significant. It usually takes a few minutes before equilibrium is established. Therefore it is sometimes observed that enzymic hydrolysis increases during the initial period.

In view of the instability of the carboxylic acid anhydrides in buffer of pH 7·3, it was not feasible to measure the distribution coefficient for the system toluene: water. The amount present in the aqueous phase at a given moment is thus unknown, and no quantitative comparison between different substrates can be applied.

Colorimetric methods. For the colorimetric determination of choline esters the method of Hestrin (1949) was used. Since, in spite of precautions, the samples taken from the two-phase reaction system always contained a small amount of toluene, it was found necessary to gas the solution with air for 1 min. before the addition of hydroxylamine, in order to transfer all anhydride into the aqueous layer and to cause its hydrolysis.

RESULTS

Enzymic hydrolysis of carboxylic acid anhydrides. Enzymic hydrolysis could not be measured when acetic or propionic anhydride was added directly to the aqueous phase, since the spontaneous decomposition of the substrate was too rapid. We therefore devised a new technique, in which the anhydride is dissolved in toluene and delivered gradually to the aqueous phase. In this two-phase technique spontaneous hydrolysis is proceeding at a relatively low rate. Even this improvement did not enable us to demonstrate unequivocally the enzymic splitting of acetic anhydride, but in the case of the higher homologues clear evidence for enzymic catalysis was obtained (Figs. 1 and 2). Although a strictly quantitative comparison is impossible, our results indicate that the order of reactivity is probably analogous to that of the corresponding esters. For example, butyric anhydride is not attacked by cholinesterase, but is a good substrate for liver esterase. It may also be mentioned that cholinesterase does not attack an aqueous solution of ethyl propionate when the substrate concentration is equal to that which would be obtained from the corresponding anhydride if the latter could pass quantitatively into the aqueous phase. For acetylcholinesterase, propionic anhydride is clearly a better substrate than ethyl propionate.

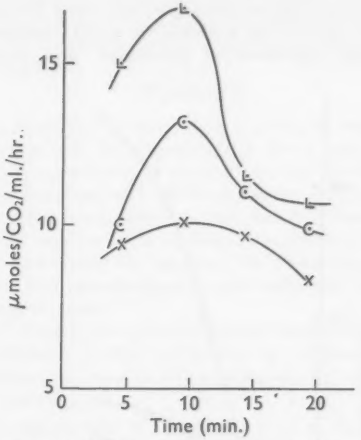


Fig. 1. Enzymic hydrolysis of propionic anhydride at 23°. The anhydride was dissolved in toluene, 1:100, and 0·2 ml. of this solution put into the side bulb of a Warburg vessel. The thermobarometer contained toluene only. The main compartment received either buffer alone, or a buffered enzyme solution, 2·9 ml. ×—×, spontaneous hydrolysis; ⊙—⊙, hydrolysis by *Torpedo* esterase, 1:2500; ⌊·—⌊·, hydrolysis by liver esterase, 1:60.

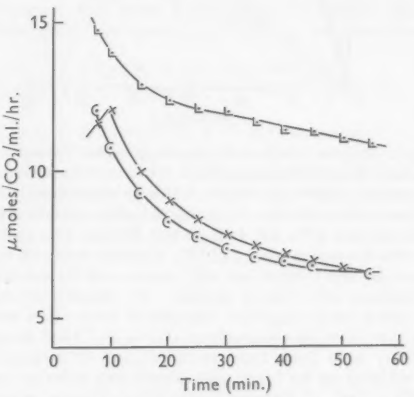


Fig. 2. Enzymic hydrolysis of butyric anhydride at 23°. The anhydride was dissolved in toluene, 1:10, and 0·3 ml. of this solution put into the side bulb, whereas the main compartment contained 2·8 ml. of buffer or buffered enzyme. ×—×, spontaneous hydrolysis; ⊙—⊙, hydrolysis by *Torpedo* esterase, 1:2500; ⌊·—⌊·, hydrolysis by liver esterase, 1:60.

Enzymic synthesis of choline esters from anhydrides. It was shown first by Hestrin (1950) that cholinesterase is able to synthesize esters from choline and fatty acids, and similar experiments on the system choline-fatty acid ester have been carried out by Wilson, Bergmann & Nachmansohn (1950). Applying our two-phase technique, and using the colorimetric method of analysis, we could demonstrate enzymic synthesis of propionyl- and butyrylcholine from the respective anhydrides (Figs. 3 and 4). Comparison of the rates of ester synthesis from propionic anhydride and propionic acid shows that the former substrate reacts much faster, thus excluding the possibility that the anhydride is hydrolysed before condensation with choline. Thus Hestrin (1950) used both choline and propionate in about 1·5M solution, and acetylcholinesterase in a concentration sufficient to hydrolyse 2 g. acetylcholine/ml./hr. under optimum conditions. He obtained, however, not more than about 0·1 μmole propionylcholine/ml./hr. In the experiment of Fig. 3 we worked with an enzyme concentration which hydrolysed 27 mg. acetylcholine/ml./hr.; using 0·15M choline, we observed the formation of 8 μmoles of propionylcholine/ml./hr. This is evidence that the enzyme catalyses directly the condensation of the anhydride with choline.

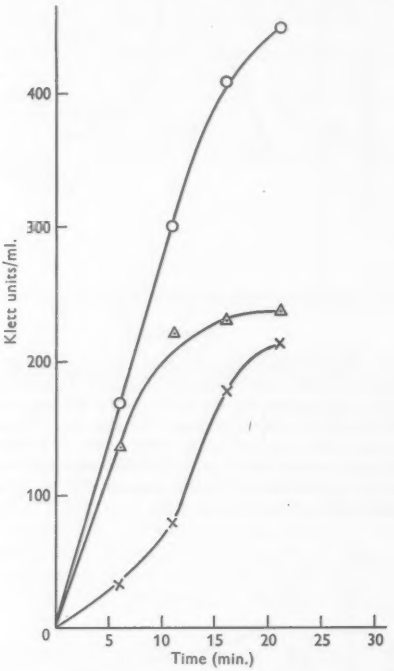


Fig. 3. Enzymic synthesis of propionylcholine. The aqueous phase of each Erlenmeyer flask, 8 ml., contained *Torpedo* esterase, 1:1600, and choline, 0·15M, in bicarbonate buffer (see under methods). Propionic anhydride was dissolved in toluene, 1:50, and 4 ml. of this dilution were shaken with the aqueous layer at 23°. Controls were run with choline and toluene, and with choline and the substrate solution, but without enzyme. All experiments were carried out in duplicate. Samples of 0·5 ml. were withdrawn from the aqueous layer after 5, 10, 15 and 20 min. They were freed from small amounts of toluene by bubbling air for 1 min. and diluted with water to 1 ml. Then 2 ml. of Hestrin's (1949) hydroxylamine mixture were added, and, after 1 min. incubation, the solution was acidified with 1 ml. of 3·5N-HCl and the coloured complex was developed with 1 ml. of 0·74M-$FeCl_3$, dissolved in 0·1N-HCl. Readings were taken with a Klett-Summerson photoelectric colorimeter, using filter no. 54. ×—×, control; ○—○, enzymic reaction; △—△, difference between enzymic and spontaneous reaction.

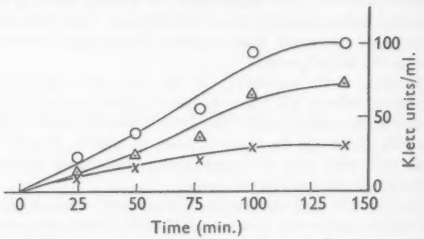


Fig. 4. Enzymic synthesis of butyrylcholine. Conditions as for Fig. 3. Butyric anhydride was dissolved in toluene 1:10. Samples were withdrawn after 25, 50, 77, 100 and 140 min. ×—×, control; ○—○, enzymic reaction; △—△, difference between enzymic and non-enzymic esterification.

The behaviour of a mixed anhydride towards cholinesterase. On the basis of these results it could be predicted that a mixed phosphoric-carboxylic anhydride would also be split by esterases. This type of substrate appeared of special interest since, depending on the preferred direction of hydrolysis, it could serve either as substrate or as inhibitor. We found that dibenzyl acetyl phosphate, at a concentration of 4×10^{-5}M, produced 50% inhibition of acetylcholinesterase, and that this effect was irreversible. However, when the spontaneous hydrolysis of the mixed anhydride was followed by the method of Lipmann & Tuttle (1945), it was found that after complete hydrolysis in Warburg buffer at pH 7·3 (about 20 min.) the inhibitory effect was practically unaltered, indicating that a second compound with a lower rate of hydrolysis may be responsible for the inhibition. The observations of Sheehan & Frank (1950) on related anhydrides suggest that this compound is probably tetrabenzyl pyrophosphate, formed by disproportionation of dibenzyl acetyl phosphate upon standing. Indeed, tetrabenzyl pyrophosphate is

several hundred times more effective than the mixed anhydride, its I_{50} value being about 1×10^{-7} M. A rather small degree of disproportionation would therefore be sufficient to explain our results. Therefore we cannot sustain the claim made by us in a preliminary communication (Bergmann, Wurzel & Shimoni, 1953) that dibenzyl acetyl phosphate is itself an inhibitor of esterases.

DISCUSSION

Our experiments support the view that various derivatives of phosphoric and carboxylic acids are treated principally in the same way by esterases. The demonstration of enzymic hydrolysis of carboxylic acid anhydrides met with great difficulties until the two-phase technique was introduced. It has been claimed recently (Wilson, 1952) that enzymic splitting of acetic anhydride could be demonstrated in the aqueous phase by sampling up to 30 sec. after addition of the substrate. All our own attempts during the last 2 years to measure the hydrolysis of anhydrides by the direct method have failed.

The hydrolysis of carboxylic acid anhydrides by specific and unspecific esterases supports the common mechanism of enzymic ester hydrolysis, which we proposed earlier (Bergmann *et al.* 1950), namely that the electrophilic carbon of the acylinium part ($R\overset{+}{C}O$—) of anhydride or ester combines with the nucleophilic group of the active surface, while the rest of the molecule is removed by combination with a hydrogen atom (probably derived from the active surface). Any compound capable of acylating the enzyme should therefore serve as substrate, e.g. acid chlorides. This question is now under investigation.

SUMMARY

1. Enzymic hydrolysis of carboxylic acid anhydrides can be demonstrated by a two-phase technique, whereby a toluene solution of the substrate is shaken with the aqueous enzyme phase.

2. Acetylcholinesterase catalyses the esterification of choline with carboxylic acid anhydrides. This was shown by applying the two-phase technique, and measuring colorimetrically the amount of ester formed.

3. These results give additional evidence for the mechanism of ester hydrolysis by esterases: the enzyme is acylated and this intermediate is split by the solvent.

The authors wish to thank Prof. Bacci of the Marine Biological Station of Naples for a gift of *Torpedo marmorata* and Dr G. W. Kenner for a small sample of tetrabenzyl pyrophosphate.

REFERENCES

Aldridge, W. N. & Davison, A. N. (1952). *Biochem. J.* **51**, 62.
Bentley, R. (1948). *J. Amer. Chem. Soc.* **70**, 2183.
Bergmann, F. & Shimoni, E. (1952). *Biochim. biophys. Acta*, **8**, 520.
Bergmann, F., Wilson, I. B. & Nachmansohn, D. (1950). *J. biol. Chem.* **186**, 693.
Bergmann, F., Wurzel, M. & Shimoni, E. (1953). *Nature, Lond.*, **171**, 744.
Burgen, A. S. V. (1949). *Brit. J. Pharmacol.* **4**, 219.
Hestrin, S. (1949). *J. biol. Chem.* **180**, 249.
Hestrin, S. (1950). *Biochim. biophys. Acta*, **4**, 310.
Kenner, G. W., Todd, A. R. & Weymouth, F. J. (1952). *J. chem. Soc.* p. 3675.
Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **161**, 415.
Sheehan, J. C. & Frank, V. S. (1950). *J. Amer. chem. Soc.* **72**, 1312.
Wilson, I. B. (1952). *J. biol. Chem.* **197**, 215.
Wilson, I. B. & Bergmann, F. (1950). *J. biol. Chem.* **185**, 479.
Wilson, I. B., Bergmann, F. & Nachmansohn, D. (1950). *J. biol. Chem.* **186**, 781.

The Possible Role of Coenzyme A in the Biosynthesis of Cholesterol in the Rat

By G. S. BOYD
Department of Biochemistry, University of Edinburgh

(*Received* 23 *December* 1952)

With the aid of isotopes it has been established by *in vivo* experiments that mammals can synthesize cholesterol from acetate (Bloch & Rittenberg, 1942); and by the tissue-slice technique various mammalian tissues have been shown to be capable of cholesterol biosynthesis from acetate *in vitro* (Bloch, Borek & Rittenberg, 1946; Srere, Chaikoff, Treitman & Burstein, 1950). In such *in vitro* experiments it has been demonstrated that, in this biosynthesis, both carbon atoms of acetate are extensively incorporated into both the *cyclo*pentenophenanthrene ring system of the molecule, and into the *iso*octyl side chain (Bloch & Rittenberg, 1942; Bloch, 1951).

Nevertheless, there still exists a huge gap in our knowledge regarding the precise steps involved in the use of acetate in the biosynthesis of this complex C_{27} molecule. To date, possibly the only established intermediate in this process is acetoacetate, as it has been shown by *in vitro* studies that this compound could be incorporated into cholesterol without prior degradation to acetate (Brady & Gurin, 1951). This work suggests, therefore, that a stage in the biosynthesis of cholesterol might involve the condensation of two acetate molecules.

It has been known for some time that co-factors are required in simple biological acetylation reactions such as the acetylation of choline (Feldberg & Mann, 1945; Lipton & Barron, 1946; Nachmansohn & Berman, 1946) or in the acetylation of a foreign amine, for example sulphanilamide (Lipmann, 1945), while the co-factors necessary in the acetylation of both alcohols and amines were shown to be identical by Lipmann & Kaplan (1946). Soodak & Lipmann (1948) showed that this coenzyme was also involved in the reaction acetate plus acetate yielding acetoacetate. Lipmann, Kaplan, Novelli, Tuttle & Guirard (1947) demonstrated that this co-acetylase factor (coenzyme A) contains pantothenic acid in a bound form. This coenzyme was isolated in fairly pure form by Lipmann, Kaplan, Novelli, Tuttle & Guirard (1950) and its structure established by the joint efforts of many workers including Lipmann *et al.* (1950); Novelli, Kaplan & Lipmann (1950); Baddiley & Thain (1951*a*, *b*, *c*).

According to Lynen, Reichert & Rueff (1951), coenzyme A possibly participates in biological acetylations by accepting an acetyl radical at its terminal sulphydryl group, producing a thioacetyl coenzyme and transferring this 'energy-rich' acetyl group to the substrate.

It has been shown that the coenzyme-A content is lower than normal in tissues obtained from a bird or mammal which has been fed a diet deficient in pantothenate (Olson & Kaplan, 1948; Olson & Stare, 1951) and consequently the ability of an animal to effect acetylations of the types previously mentioned is markedly reduced in this deficiency state.

It seemed of interest to attempt to establish whether the multiple utilization of acetate by mammals in the biosynthesis of cholesterol requires the participation of coenzyme A and this paper deals with the experimental details and implications of work on this problem previously reported in the form of an Abstract (Boyd, 1952).

METHODS

Preparation of animals. The animals used in this study were male and female albino rats of the Wistar strain. After weaning, the rats were placed in wire-mesh metabolism cages in groups of four to six, and allowed tap water *ad libitum*. The diet consisted of approx. 10 g./day/rat of the following basal ration: 23% vitamin-free casein, supplemented with 0·2% cystine; 72% carbohydrate (glucose or sucrose); 5% salt mixture (Phillips & Hart, 1935) containing 0·05% $CoCl_2$, $6H_2O$.

Water-soluble vitamins were added to the diet in the following amounts (per 100 g. basal ration), which are recognized as adequate for the growing rat: thiamine hydrochloride, 500 μg.; riboflavin, 800 μg.; nicotinamide, 4 mg.; pyridoxine hydrochloride, 500 μg.; biotin, 40 μg.; folic acid, 200 μg.; menaphthone (2-methyl-1:4-naphthaquinone), 500 μg.; inositol, 10 mg.; *p*-aminobenzoic acid, 10 mg.; choline chloride, 20 mg.; calcium pantothenate, 3 mg.

The pantothenate-deficient groups, who were litter mates of the control groups, received the diet exactly as above except that they were deprived of pantothenate. All groups received 1 ml. cod-liver oil/5 rats/week, in two divided doses, which is sufficient to supply the fat-soluble vitamins and the 'essential' fatty acids.

Where certain groups of rats received neutral fat (olive oil) in the diet, this was added at a 10·5% level, while a corresponding reduction was made in the carbohydrate content in order to keep the diets isocaloric. Thus in the experiments to be reported in this paper, there were four groups of rats on the following diets: (*a*) fat-replete, pantothenate-replete, (F+, P+); (*b*) fat-replete, pantothenate-deficient, (F+,

P−); (*c*) fat-deficient, pantothenate-replete, (F−, P+); (*d*) fat-deficient, pantothenate-deficient, (F−, P−).

The caloric intakes of all groups were matched day by day; the animals were fed twice daily, at 9 a.m. and 9 p.m., and weighed each morning.

The pantothenate-deficient group showed all the external symptoms of this deficiency state, such as thinning of fur and porphyrin-stained whiskers, etc.; furthermore, after 10 days on this regimen the animals had virtually ceased growing. Selected pantothenate-deficient animals were then given calcium pantothenate in their diet, whereupon they resumed normal growth and were soon free from all deficiency symptoms; these 'test animals' were then discarded.

The growth curves of two groups of animals (F−, P+ and F−, P−) are shown in Fig. 1, in which the response of the pantothenate-deficient animals to this factor is shown.

After 40 days on one of the diets previously specified, each animal was anaesthetized with Nembutal (approx. 0·05 ml./100 g. body weight) and the carotid artery exposed. As much blood as possible was removed by cannulation of this vessel into a centrifuge tube containing a minimum of powdered potassium oxalate.

Plasma cholesterol estimation. Samples of the plasma (1 ml.) were extracted with about 15 ml. boiling acetone:ethanol (1:1) in a 25 ml. volumetric flask for a few minutes. The extracts were then cooled, made up to the mark and filtered. Portions of this solution were taken for estimation of total and free cholesterol by the Sperry-Schoenheimer digitonin procedure as modified by Sperry & Webb (1950).

Liver cholesterol estimation. After the death of the animal by exsanguination, the liver was completely removed, blotted on filter paper and weighed. This tissue was ground with an equal wt. of distilled water in an all-glass homogenizer (Potter & Elvehjem, 1936), samples of the suspension were extracted with $CHCl_3$:methanol (1:1, v/v) and cholesterol was estimated in exactly the same manner as in the plasma cholesterol determination.

Liver coenzyme A. Samples of the liver suspension were delivered at once into about 30 ml. boiling distilled water in a Pyrex centrifuge tube, and the resulting suspension was kept at boiling point for one minute. The tube was then chilled in ice-water, centrifuged, the supernatant decanted into a 50 ml. graduated vessel and made up to volume with distilled water. These aqueous extracts were stored at −10° until the coenzyme-A assays could be performed. The assay procedure employed was that of Handschumacher, Mueller & Strong (1951), which was checked against the original sulphanilamide method of assay (Lipmann, 1945; Kaplan & Lipmann, 1948) and found to be more rapid than the latter, but yielded similar results. Standard curves, prepared by the use of reference coenzyme-A samples, were used to measure the coenzyme-A concentrations of the aqueous liver extracts (Kaplan & Lipmann, 1948).

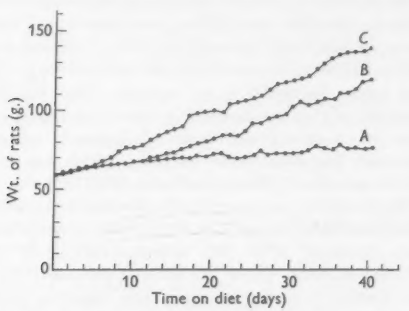


Fig. 1. Growth curves of groups of rats on diets as follows: *A*, pantothenate-deficient; *B*, pantothenate-deficient for 10 days and then pantothenate-replete; *C*, pantothenate-replete.

RESULTS

The results of these observations are summarized in Table 1. When pantothenate was withheld from the diet of a group of young rats for 40 days, the coenzyme-A content of the livers of these animals dropped to a value less than half that found in the control groups of pantothenate-replete animals.

In all four groups of animals dietary restriction failed to alter significantly the level of free cholesterol in the plasma, but the free cholesterol of liver in one group (fat-deficient, pantothenate-deficient) underwent a slight decrease.

If the two fat-deficient groups are compared, it is seen that withholding pantothenate from one group

Table 1. *Comparison of plasma and liver cholesterol, and liver coenzyme A in control and pantothenate-deficient rats* (*means* ± S.D.)

Diet	Days on diet	No. of rats	Plasma cholesterol (mg./100 ml.) Total	Free	Ester	Liver cholesterol (mg./100 g. wet wt.) Total	Free	Ester	Liver cholesterol (mg./liver)	Liver coenzyme A (units/g. wet wt.)
Fat-deficient, pantothenate-replete	40	12	64±8·0	17±2·3	47±6·0	248±8·0	183±14	65±14	22·2±2·3	109±17
Fat-deficient, pantothenate-deficient	40	12	44±5·9	16±2·2	28±4·6	181±13	168±10	13±8	16·1±1·8	49±19
Fat-replete, pantothenate-replete	40	12	68±7·7	18±3·5	50±4·7	368±73	205±13	163±63	42·5±7·1	123±21
Fat-replete, pantothenate-deficient	40	12	71±10	18±4·0	53±6·4	301±36	203±8·0	98±31	28·6±3·9	41±8

resulted in a significant drop in the plasma and liver total cholesterol. This drop was due almost entirely to a decrease in the ester cholesterol fraction, since the free cholesterol is fairly constant. Thus on a fat-deficient diet, when the coenzyme-A content of the liver (and presumably other organs) is decreased, the plasma and liver ester cholesterol concentration is reduced.

On the other hand, in the fat-replete groups, the plasma and liver total-cholesterol levels were unaffected by the presence or absence of pantothenate in the diet. Thus the animals in the fat-replete, pantothenate-deficient group maintained normal plasma and liver ester cholesterol levels despite a reduction in tissue coenzyme-A concentration.

DISCUSSION

On a fat-free diet the rat appears to be able to maintain its plasma ester cholesterol at about the normal value, although the liver ester cholesterol concentration is decreased. If fat and pantothenate are withheld from the diet, then the plasma ester cholesterol is markedly reduced and the liver ester cholesterol virtually disappears; in most rats in this group, the livers were completely devoid of any ester cholesterol. Since the animals were maintained on a sterol-free diet, nearly all the carcass cholesterol must have been of endogenous origin. Accepting the normal glycolysis mechanism, presumably most of the acetate used in the biosynthesis of cholesterol arose from glucose. Thus on the fat-free diet, pantothenate restriction resulted in a lowered tissue coenzyme-A concentration, producing a decrease in the plasma and liver ester-cholesterol concentration.

On this basis it would be tempting to suggest that the biosynthesis of cholesterol ester involved coenzyme A.

When neutral fat was incorporated in the diet, however, restriction of dietary pantothenate had no effect on the plasma ester cholesterol concentration, and little, if any, effect on the liver ester cholesterol concentration. It appeared, therefore, that the presence of neutral fat in the diet could overcome the apparent inhibition of ester cholesterol synthesis due to lowered tissue coenzyme A, as was observed in the fat-free groups.

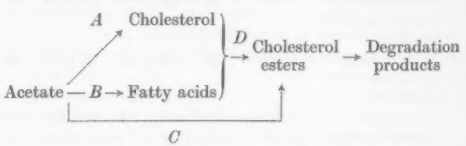
These seemingly anomalous experimental results might be explained as follows. On a fat-free diet the organism synthesizes cholesterol from acetate; thus a two-carbon compound may give rise to a four-carbon compound, such as acetoacetate, and perhaps larger even-numbered carbon compounds, as intermediates in the biosynthesis of the sterol. This process would involve the multiple utilization of acetate to produce intermediates in the biosynthesis which contain even numbers of carbon atoms. Each addition of acetate to the preceding intermediate, for instance acetate plus acetate yielding acetoacetate, would then involve coenzyme A, and at the same time produce an intermediate common to cholesterol biosynthesis and fatty acid catabolism. Thus if the tissue coenzyme-A concentration is reduced, the ability to synthesize acetoacetate, or other even-numbered carbon atom compounds, from acetate will be reduced, and if these compounds are intermediates in cholesterol biosynthesis, then this theory would explain the drop in ester cholesterol observed in the fat deficient, pantothenate-deficient group.

If neutral fat is present in the diet, then the catabolism of the fatty acids could produce C_4, C_6, and higher even-numbered carbon atom intermediates, which may be required in the biosynthesis of cholesterol. This process would in all probability reduce the tissue coenzyme-A requirement to produce a given amount of a certain intermediate. Hence the presence of fatty acids in the diet would overcome the partial inhibition of the synthesis of these intermediates due to the decreased tissue coenzyme-A content.

On this theory, based on these experimental facts, it seems possible that certain even-numbered fatty acids, or fatty acid derivatives (other than acetate), can enter the cholesterol biosynthetic pathway without prior degradation to acetate. The simplest example of this mechanism is the direct incorporation of acetoacetate into cholesterol without previous degradation to acetate, which has been shown *in vitro* by Brady & Gurin (1951).

Bloch (1951) has described experiments in which cholesterol and mixed non-volatile fatty acids have been isolated after the administration of ^{14}C-labelled acetate. Since the specific radioactivity of the former was higher than the 'specific radioactivity' of the latter, Bloch concluded that cholesterol could not be synthesized via the non-volatile fatty acids. While this conclusion may be correct, the use of data derived from the so-called 'specific radioactivity of a mixture' cannot be justified in support of this theory.

Finally, it could be argued that decreased ester cholesterol in the presence of decreased coenzyme-A concentration could be due to an interference with the cholesterol esterification reaction. The following argument would appear to dismiss this hypothesis.



1. Cholesterol is synthesized from acetate (reaction *A*).
2. Fatty acids are synthesized from acetate (reaction *B*).
3. Ester cholesterol is synthesized from cholesterol plus fatty acids (reaction *D*).

Hence ester cholesterol is synthesized from acetate, and this work suggests that the biosynthesis of ester cholesterol is dependent on coenzyme A.

If the reduction in ester cholesterol due to decreased coenzyme A were mediated through reaction *D*, and the biosynthesis of cholesterol proceeded normally when the coenzyme-A level was lower than usual, then there should be a corresponding increase in free cholesterol. This was not observed.

If the reaction *D* were the point at which the blockage in ester cholesterol biosynthesis occurred due to decreased coenzyme A, then overcoming this inhibition by fatty acids (presumably by a 'mass action effect') as observed by the author should result in an increase in ester cholesterol at the expense of the free cholesterol. This was not observed.

Hence it can be deduced that reaction *A*, the synthesis of cholesterol from acetate appears to be coenzyme-A dependent.

SUMMARY

1. It has been observed that if rats are maintained on a fat-free diet, the liver ester cholesterol concentration and the plasma ester cholesterol concentration appear to be dependent on dietary pantothenate and hence on the coenzyme-A content of the tissues.

2. The presence of neutral fat in the diet can annul these effects.

3. A possible explanation of these results is submitted.

This work is part of a programme of research undertaken on behalf of the Coronary Thrombosis Study Group of the Standing Medical Advisory Committee (Scotland). The author wishes to thank the Advisory Committee on Medical Research (Scotland) for a grant from which the expenses of the work were defrayed.

REFERENCES

Baddiley, J. & Thain, E. M. (1951*a*). *Chem. & Ind.* p. 337.
Baddiley, J. & Thain, E. M. (1951*b*). *Chem. & Ind.* p. 1003.
Baddiley, J. & Thain, E. M. (1951*c*). *J. chem. Soc.* p. 246.
Bloch, K. (1951). *Recent Progr. Hormone Res.* **6**, 111.
Bloch, K., Borek, E. & Rittenberg, D. (1946). *J. biol. Chem.* **162**, 441.
Bloch, K. & Rittenberg, D. (1942). *J. biol. Chem.* **145**, 625.
Boyd, G. S. (1952). *2nd Int. Congr. Biochem. Abstr.*, p. 120.
Brady, R. O. & Gurin, S. (1951). *J. biol. Chem.* **189**, 371.
Feldberg, W. & Mann, P. J. G. (1945). *J. Physiol.* **104**, 17*P*.
Handschumacher, R. E., Mueller, G. C. & Strong, F. M. (1951). *J. biol. Chem.* **189**, 335.
Kaplan, N. O. & Lipmann, F. (1948). *J. biol. Chem.* **174**, 37.
Lipmann, F. (1945). *J. biol. Chem.* **160**, 173.
Lipmann, F. & Kaplan, N. O. (1946). *J. biol. Chem.* **162**, 743.
Lipmann, F., Kaplan, N. O., Novelli, G. D., Tuttle, L. C. & Guirard, B. M. (1947). *J. biol. Chem.* **167**, 869.
Lipmann, F., Kaplan, N. O., Novelli, G. D., Tuttle, L. C. & Guirard, B. M. (1950). *J. biol. Chem.* **186**, 235.
Lipton, M. A. & Barron, E. S. G. (1946). *J. biol. Chem.* **166**, 367.
Lynen, F., Reichert, E. & Rueff, L. (1951). *Liebigs Ann.* **547**, 1.
Nachmansohn, D. & Berman, M. (1946). *J. biol. Chem.* **165**, 551.
Novelli, G. D., Kaplan, N. O. & Lipmann, F. (1950). *Fed. Proc.* **9**, 209.
Olson, R. E. & Kaplan, N. O. (1948). *J. biol. Chem.* **175**, 515.
Olson, R. E. & Stare, F. J. (1951). *J. biol. Chem.* **190**, 149.
Phillips, P. H. & Hart, E. B. (1935). *J. biol. Chem.* **109**, 657.
Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.
Soodak, M. & Lipmann, F. (1948). *J. biol. Chem.* **175**, 999.
Sperry, W. M. & Webb, M. (1950). *J. biol. Chem.* **187**, 97.
Srere, P. A., Chaikoff, I. L., Treitman, S. S. & Burstein, L. S. (1950). *J. biol. Chem.* **182**, 629.

Index of Authors

Note. Entries marked with an asterisk (*) refer to communications made in title only.
Entries marked with a section mark (§) refer to contributions to Symposia.
Small roman numerals refer to pages in *Proceedings of the Biochemical Society.*

AHMAD, K. & KARIM, M. A. Biosynthesis of choline in the seedling of the chick-pea (*Cicer arietinum*) 817

AITKEN, E. H. & PREEDY, J. R. K. The presence of potassium as a source of inaccuracy in the chemical estimation of sodium in urine 211

ALDRIDGE, W. N. & DAVISON, A. N. The mechanism of inhibition of cholinesterases by organophosphorus compounds 763

ALMOND, A. E., FRANCIS, G. E., HAWKINS, J. D. & WORMALL, A. The fate of intravenously injected ^{131}I-labelled antigens and 3:5-diiodotyrosine in immune and normal rabbits xxxiii

ANSELL, G. B. & NORMAN, J. M. Glycerylphosphorylethanolamine in rat brain 768

ARNSTEIN, H. R. V. & CRAWHALL, J. C. The metabolism of DL-[β-^{14}C] and DL-[^{35}S]cystine by the rat 280

ARNSTEIN, H. R. V. & GRANT, P. T. The biosynthesis of penicillin v

ARNSTEIN, H. R. V. & NEUBERGER, A. The effect of cobalamin on the quantitative utilization of serine, glycine and formate for the synthesis of choline and methyl groups of methionine 259

ARNSTEIN, H. R. V. & NEUBERGER, A. The synthesis of glycine and serine by the rat 271

ATERMAN, K., BOSCOTT, R. J. & COOKE, W. T. The significance of urinary *p*-hydroxyphenylacetic acid (PHPA) xvii

AYRES, P. J. W. & MCILWAIN, H. Techniques in tissue metabolism. 2. Application of electrical impulses to separated tissues in aqueous media 607

AZOUZ, W. M., PARKE, D. V. & WILLIAMS, R. T. Studies in detoxication. 51. The determination of catechols in urine, and the formation of catechols in rabbits receiving halogenobenzenes and other compounds. Dihydroxylation *in vivo* 146

BACK, K. J. C. & WOODS, D. D. Studies with a strain of *Bacterium coli* requiring citrulline and a pyrimidine for growth xii

BAKER, J. W. A possible model of the tryptophan-tryptophanase action xxxii

BALDWIN, R. L. Sedimentation coefficients of small molecules: methods of measurement based on the refractive-index gradient curve. The sedimentation coefficient of polyglucose A 644

BARLOW, A. J. E., CHATTAWAY, F. W. & THOMPSON, C. C. Effect of inhibitors on the metabolism of *Microsporum* xxxi

BARNETT, A. J. G. & DUNCAN, R. E. B. The formation of volatile fatty acids in laboratory mixtures of minced crops and water, and the occurrence of these acids in field silage xix

BARNETT, A. J. G. *see also* ROBERTSON, H. A. xx

BARRY, J. M. *see* REISS, O. K. 783

BARTLEY, W. *see* HEMS, R. 434

BARUAH, P. & SWAIN, T. The effect of L-ascorbic acid on the *in vitro* activity of polyphenoloxidase from potato 392

BAULD, W. S. *see* KERR, L. M. H. 872

BEALING, F. J. Mould 'glucosaccharase': a fructosidase 93

BENSON, A. *see* FALK, J. E. 101

BERGMANN, F. & SHIMONI, A. The enzymic hydrolysis of alkyl fluoroacetates and related compounds 50

BERGMANN, F., WURZEL, M. & SHIMONI, E. The enzymic hydrolysis of acid anhydrides 888

BHATIA, I. S. *see* SRINIVASAN, M. 286

BICHOWSKI-SLOMNITZKI, L. *see* KATCHALSKI, E. 671

EIDWELL, E. Fibrinolysins of human plasma. A comparison of fibrinolytic plasma from normal subjects and from cadaver blood with plasmin prepared by activation with chloroform 497

BIERI, J. G. & POLLARD, C. J. The site of conversion of carotene injected into rats xxxi

BILLING, B. H. *see* COLE, P. G. xiii

BOSCOTT, R. J. & GREENBERG, S. M. The effect of dietary deficiencies, aminopterin, ascorbic acid, chloretone and antibiotics on the urinary excretion of phenolic acids in the rat xviii

BOSCOTT, R. J. *see also* ATERMAN, K. xvii

BOURSNELL, J. C., COOMBS, R. R. A. & RIZK, V. Studies with marked antisera. Quantitative studies with antisera marked with ^{131}iodine isotope and their corresponding red-cell antigens 745

BOWEN, T. J. Physical studies on a soluble protein obtained by the degradation of elastin with urea 766

BOWES, J. H. & MOSS, J. A. The reaction of fluorodinitrobenzene with the α- and ϵ-amino groups of collagen 735

BOYD, G. S. The possible role of coenzyme A in the biosynthesis of cholesterol in the rat 892

BROADBENT, I. E., BROOKS, R. V. & KLYNE, W. Some lipid fractions from the urines of bulls and pregnant cows vi

BROOKS, C. J. W. & NORYMBERSKI, J. K. The oxidation of corticosteroids with sodium bismuthate 371

BROOKS, R. V. *see* BROADBENT, I. E. vi

BROUWER, E. & NIJKAMP, H. J. Occurrence of two valeric acids (β-methylbutyric acid and α-methylbutyric acid) in the hair grease of the dog 444

BROWNIE, A. C., GRANT, J. K. & TAYLOR, W. Enzymic 11-β-hydroxylation of 11-deoxycorticosterone (DOC) xxii

CALDWELL, P. C. The separation of the phosphate esters of muscle by paper chromatography 458

CAMA, J. S. & MEARA, M. L. The component acids of milk fats of *Balaenoptera physalus* (finner whale) 365

CANIVET, J. & RIMINGTON, C. A chromatographic study of uroporphyrins from cases of cutaneous, acute and congenital porphyria 867

CASPARY, E. A. Physicochemical examination of a mucoid from the salivary gland of sheep. *Addendum to* Studies on influenza virus receptor-substance and receptor-substance analogues. 1. Preparation and properties of a homogeneous mucoid from the salivary gland of sheep (McCrea, J. F.) 139

CHALMERS, J. G. The role of soluble proteins in the elimination of carcinogens from the animal body xix

CHAPPELL, J. B. & PERRY, S. V. The respiratory and adenosinetriphosphatase activities of skeletal-muscle mitochondria 586

CHATTAWAY, F. W. *see* BARLOW, A. J. E. xxxi

CHEN, Y.-T., ISHERWOOD, F. A. & MAPSON, L. W. Quantitative estimation of ascorbic acid and related substances in biological extracts by separation on a paper chromatogram 821

Chibnall, A. C., Piper, S. H. & Williams, E. F. Further observations on phrenosinic acid 707

Chibnall, A. C., Piper, S. H. & Williams, E. F. The α-hydroxy-*n*-fatty acids of yeast cerebrin 711

Clark, N. G. & Hams, A. F. The fungicidal activity of substituted acetanilides and related compounds 839

Clarke, G. D. *see* Clarke, W. A. xvi

Clarke, W. A. & Clarke, G. D. The mechanism of the effect of iron and cobalt on the production of toxin by *Corynebacterium diphtheriae* xvi

Cleland, K. W. *see* Slater, E. C. 566

Cohen, S. S. *see* McNair Scott, D. B. 23, 33; Wyatt, G. R. 774

Cole, D. F. Action of deoxycorticosterone acetate and cortisone on response of adrenalectomized rats to hypotonic load xiii

Cole, P. G., Lathe, G. H. & Billing, B. H. The diazo reacting pigments of serum, urine and bile xiii

Cole, P. G., Lathe, G. H. & Ruthven, C. R. J. The application of countercurrent methods to the fractionation of lipid material from human placenta 17

Conchie, J. Inhibition of rumen β-glucosidase activity by glucono-1:5-lactone xxi

Connell, J. J. Studies on the proteins of fish skeletal muscle. 2. Electrophoretic analysis of low ionic strength extracts of several species of fish 378

Consden, R., Glynn, L. E. & Stanier, W. M. A chemical examination of connective tissue in rheumatic fever 248

Conway, E. J. & Hingerty, D. A comparison of the effects of cortisone and deoxycorticosterone on various aspects of organic and inorganic metabolism 447

Conway, E. J. & Hingerty, D. The effects of cortisone, deoxycorticosterone and other steroids on the active transport of sodium and potassium ions in yeast 455

Cooke, W. T. *see* Aterman, K. xvii

Coombs, R. R. A. *see* Boursnell, J. C. 745

Corfield, M. C. & Robson, A. The amino acid composition of salmine 517

Coxon, R. V. The accumulation of citrate during oxidation of pyruvate by breis and slices of pigeon brain 545

Crawhall, J. C. & Elliott, D. F. The preparation and properties of DL-cysteinol and some of its derivatives vii

Crawhall, J. C. *see also* Arnstein, H. R. V. 280

Cromwell, B. T. & Rennie, S. D. The biosynthesis and metabolism of betaines in plants. 1. The estimation and distribution of glycinebetaine (betaine) in *Beta vulgaris* L. and other plants 189

Crook, E. M. & Stone, B. A. Formation of oligosaccharides during the enzymic hydrolysis of β-glucosides xxv

Cuthbertson, W. F. J., Ireland, D. M. & Wolff, W. Detection and identification of some metabolites of *iso*-nicotinic acid hydrazide (isoniazid) in human urine 669

Dagley, S. & Dawes, E. A. Dissimilation of citric acid by extracts of *Aerobacter aerogenes* xvi

Dagley, S. & Dawes, E. A. Enzymic estimation of citric acid xxxv

Dagley, S. & Patel, M. D. Production of keto acids from acetate by a vibrio xxxvi

Dalziel, K. An apparatus for the spectrokinetic study of rapid reactions 79

Dalziel, K. The precision of analysis by photoelectric spectrophotometry. *Appendix to* An apparatus for the spectrokinetic study of rapid reactions (Dalziel, K.) 90

Davies, R. Enzyme formation in *Saccharomyces fragilis*. 1. Invertase and raffinase 484

Davies, R. E. *see* Kornberg, H. L. ii, iii; Whittam, R. 880

Davison, A. N. *see* Aldridge, W. M. 763

Dawes, E. A. *see* Dagley, S. xvi, xxxv

Dawson, R. M. C. A complete fractionation of the cerebral phospholipids labelled with ^{32}P xii

Dawson, R. M. C. The incorporation of labelled phosphate into the lipids of a brain dispersion 507

Deluca, H. A., Rossiter, R. J. & Strickland, K. P. Incorporation of radioactive phosphate into the nucleic acids of brain slices 193

Dixon, M. The determination of enzyme inhibitor constants 170

Dixon, M. The effect of pH on the affinities of enzymes for substrates and inhibitors 161

Dodgson, K. S., Lewis, J. I. M. & Spencer, B. Studies on sulphatases. 3. The arylsulphatase and β-glucuronidase of marine molluscs 253

Dodgson, K. S. & Spencer, B. Studies on sulphatases. 4. Arylsulphatase and β-glucuronidase concentrates from limpets 315

Dodgson, K. S. & Spencer, B. Studies on sulphatases. 5. The determinations of inorganic sulphate in the study of sulphatases 436

Done, J. *see* Fowden, L. 548

Duncan, R. E. B. *see* Barnett, A. J. G. xix

Duncombe, W. G. & Glascock, R. F. Observation on the oxidation of glucose and acetate by lactating sheep mammary tissue *in vitro* xxiii

Eaton, J. C. & Gardner, M. D. Separation of cerebrospinal fluid proteins by paper electrophoresis xxv

Elliott, D. F. *see* Crawhall, J. C. vii

Elsden, S. R. & Lewis, D. The production of fatty acids by a Gram-negative coccus 183

Elsden, S. R. & Ormerod, J. G. The action of monofluoroacetate on the metabolism of *Rhodospirillum rubrum* iii

Ennor, A. H. & Stocken, L. A. The application of the diacetyl reaction to the estimation of creatine in urine 310

Evans, W. C. *see* Simpson, J. R. xxiv

Falk, J. E. & Benson, A. Separation of uroporphyrin esters I and III by paper chromatography 101

Fernell, W. R. & King, H. K. The chemical composition of the soluble and insoluble fractions of the bacterial cell 758

Findlay, M., Rossiter, R. J. & Strickland, K. P. Factors affecting the incorporation of radioactive phosphate into the pentosenucleic acids of brain slices 200

Fischer, M. I. *see* Mawson, C. A. 696

Folkes, B. F. *see* Yemm, E. W. 700

Folkes, J. P. *see* Gale, E. F. 721, 730, xi

Fowden, L. & Done, J. A third unsaturated amino acid in groundnut plants: evidence for the occurrence of γ-amino-α-methylenebutyric acid 548

Fowweather, F. S. The detection of galactose in urine 718

Francis, G. E. *see* Almond, A. E. xxxiii

Francis, J., Macturk, H. M., Madinaveitia, J. & Snow, G. A. Mycobactin, a growth factor for *Mycobacterium johnei*. 1. Isolation from *Mycobacterium phlei* 596

Friedler, L. & Smith, J. N. Hippuric acid formation in the locust vi

Gaitonde, M. K. & Richter, D. Protein synthesis in the rat brain viii

Gale, E. F. & Folkes, J. P. Amino acid incorporation by fragmented staphylococcal cells xi

Gale, E. F. & Folkes, J. P. The assimilation of amino acids by bacteria. 18. The incorporation of glutamic acid into the protein fraction of *Staphylococcus aureus* 721

Gale, E. F. & Folkes, J. P. The assimilation of amino acids by bacteria. 19. The inhibition of phenylalanine incorporation in *Staphylococcus aureus* by chloramphenicol and *p*-chlorophenylalanine 730

Gardner, M. D. *see* Eaton, J. C. xxv

GEORGE, P. The chemical nature of the second hydrogen peroxide compound formed by cytochrome *c* peroxidase and horseradish peroxidase. 2. Formation and decomposition 220
GEORGE, P. & HANANIA, G. A spectrophotometric study of ionizations in methaemoglobin 236
GEORGE, P. & IRVINE, D. H. The reaction between metmyoglobin and alkyl hydroperoxides 230
GIBBONS, R. A. *see* O'DEA, J. F. 580
GLASCOCK, R. F. *see* DUNCOMBE, W. G. xxiii
GLOCK, G. E. & MCLEAN, P. Further studies on the properties and assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver 400
GLYNN, L. E. *see* CONSDEN, R. 248
GOODER, H. & HAPPOLD, F. C. Tryptophan-tryptophanase reaction. Consideration of the nature of the enzyme-coenzyme-substrate complex xxxii
GOODWIN, T. W. The pigments in colour phases of the larvae of *Plusia gamma* L. (the silver-Y moth) 834
GORDON, J. J. Observations on brain phosphatases 812
GRANT, J. K. *see* BROWNIE, A. C. xxii
GRANT, P. T. *see* ARNSTEIN, H. R. V. v
GREEN, J. The effect of certain reducing agents on the anti-thrombic action of heparin xxiii
GREENBERG, S. M. *see* BOSCOTT, R. J. xviii
GREGORY, M. E. & HOLDSWORTH, E. S. The combination of some vitamin B_{12}-like compounds with sow's milk whey and 'intrinsic factor' concentrates 830, ix
GUGGENHEIM, K., LEIBOWITZ, J. & RAKOVER, D. Comparative observations on the chemical properties and biological activities of L-ascorbic and D-*iso*ascorbic acids 388

HACKMAN, R. H. & TODD, A. R. Some observations on the reaction of catechol derivatives with amines and amino acids in presence of oxidizing agents 631
HALL, D. A. Studies on the complex nature of the elastin-elastase system xxxv
HAMS, A. F. *see* CLARK, N. G. 839
HANANIA, G. *see* GEORGE, P. 236
HANES, C. S. *see* ISHERWOOD, F. A. 824
HANSEN, R. P. & SHORLAND, F. B. The branched-chain fatty acids of butterfat. 3. Further investigations on a multibranched C_{20} saturated fatty acid fraction 662
HAPPOLD, F. C. *see* GOODER, H. xxxii
HARRISON, M. F. Effect of starvation on the composition of the liver cell 204
HASLAM, R. M. & KLYNE, W. The precipitation of 3β-hydroxysteroids by digitonin 340
HAWKINS, J. B. & YOUNG, L. The fate of ^{35}S-labelled arylsulphuric acids after their administration to the rat ix
HAWKINS, J. D. *see* ALMOND, A. E. xxxiii
HEALD, K. & LONG, C. The enzymic phosphorylation of D-ribose xv
HEALD, P. J. The effect of metabolic inhibitors on respiration and glycolysis in electrically stimulated cerebral-cortex slices 625
HELE, M. P. The phosphorylation and absorption of sugars in the rat. 1. Hexokinase activity in the intestinal mucosa 857
HELE, M. P. The phosphorylation and absorption of sugars in the rat. 2. Sugar absorption *in vivo*, and its relationship to the phosphorylation of sugar *in vitro* 864
HELLYER, G. C. *see* WINTERINGHAM, F. P. W. xxxiii
HEMS, R. & BARTLEY, W. Preparation of ^{32}P-labelled adenosine triphosphate 434
HINGERTY, D. *see* CONWAY, E. J. 447, 455
HOLDSWORTH, E. S. Continuous electrophoresis on paper xiv
HOLDSWORTH, E. S. *see also* GREGORY, M. E. 830, ix
HOLIDAY, E. R. & PREEDY, J. R. K. The precision of a direct-reading flame photometer for the determination of sodium and potassium in biological fluids 214
HOLTON, F. A. *see* SLATER, E. C. 530
HUGHES, D. E. & WILLIAMSON, D. H. The deamidation of nicotinamide by bacteria 851
HULLIN, R. P. & NOBLE, R. L. The determination of lactic acid in microgram quantities 289
HUNTER, G. J. E. The oxidation of glycerol by mycobacteria 320
HUTCHISON, W. C. *see* WIKRAMANAYAKE, T. W. 640
HYDE, T. G. Nitrogen metabolism in *Pisum sativum* xxi

IRELAND, D. M. *see* CUTHBERTSON, W. F. J. 669
IRVINE, D. H. *see* GEORGE, P. 230
ISHERWOOD, F. A. & HANES, C. S. Separation and estimation of organic acids on paper chromatograms 824
ISHERWOOD, F. A. *see also* CHEN, Y.-T. 821

JACKSON, H. & THOMPSON, R. The reactions of some haemoglobin derivatives with ^{131}I-labelled *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene xxxvi
JOHNSTONE, J. H. & MITCHELL, I. L. S. The nature of the inhibition of yeast fermentation by D-glucosone xvii
JUDAH, J. D. & REES, K. R. The activation of fatty acid oxidation by kidney and liver mitochondria 664

KARIM, M. A. *see* AHMAD, K. 817
KATCHALSKI, E., BICHOWSKI-SLOMNITZKI, L. & VOLCANI, B. E. The action of some water-soluble poly-α-amino acids on bacteria 671
KENNARD, O. & RIMINGTON, C. Identification and classification of some porphyrins on the basis of their X-ray diffraction patterns 105
KENT, P. W. Chemical synthesis of hexose polymers 361
KENTEN, R. H. The oxidation of phenylacetaldehyde by plant saps 350
KERR, L. M. H. & BAULD, W. S. The chromatographic separation of free and combined plasma cholesterol 872
KING, H. K. *see* FERNELL, W. R. 758
KLYNE, W. & STOKES, W. M. The molecular rotations of triterpenoid alcohols and their derivatives xxviii
KLYNE, W. *see also* BROADBENT, I. E. vi; HASLAM, R. M. 340
KNIVETT, V. A. Phosphorylation coupled with anaerobic breakdown of citrulline x
KOGUT, M. & PODOSKI, E. P. Oxidative pathways in a fluorescent *Pseudomonas* 800
KORNBERG, H. L., DAVIES, R. E. & WOOD, D. R. The metabolism of [^{14}C] urea in cats not secreting gastric juice ii
KORNBERG, H. L., DAVIES, R. E. & WOOD, D. R. The metabolism of [^{14}C] urea in cats secreting gastric juice iii

LASCELLES, J. Porphyrin synthesis by *Rhodopseudomonas spheroides* iv
LATHE, G. H. *see* COLE, P. G. 17, xiii
LATNER, A. L. & MCEVOY-BOWE, E. Observations on the chemical nature of Castle's 'intrinsic factor' xxiii
LAWRIE, R. A. The activity of the cytochrome system in muscle and its relation to myoglobin 298
LAWRIE, R. A. The relation of energy-rich phosphate in muscle to myoglobin and to cytochrome-oxidase activity 305
LEIBOWITZ, J. *see* GUGGENHEIM, K. 388
LEWIS, D. *see* ELSDEN, S. R. 183
LEWIS, J. I. M. *see* DODGSON, K. S. 253
LEWIS, S. E. & SLATER, E. C. Oxidative phosphorylation in insect sarcosomes xxvii
LEWIS, S. E. *see also* SLATER, E. C. xxvii
LINDAN, O. & WORK, E. Experimental liver necrosis in rats. 1. Changes in liver, blood and spleen glutathione and ascorbic acid levels in dietetic liver necrosis 554

LINDAN, O. & WORK, E. Experimental liver necrosis in rats. 2. The levels of glutathione and ascorbic acid in livers subjected to acute circulatory congestion in relation to the levels found in dietetic liver necrosis 562
LONG, C. & MAGUIRE, M. F. Evidence for the structure of ovolecithin derived from a study of the action of lecithinase C xv
LONG, C. *see also* HEALD, K. xv
LOVE, R. M. Spectroscopic studies of carbohydrates. 1. The action of sulphuric acid on sugars 126
LOVEDAY, P. M. *see* WINTERINGHAM, F. P. W. xxxiii
LOVERN, J. A. & OLLEY, J. The lipids of fish. 4. The lipids extracted by an ethanol:ether mixture from haddock flesh previously extracted with acetone 686
LOWE, J. S. & MORTON, R. A. Studies in vitamin A. 26. The vitamin A-replacing effect of lard 681
LUNT, E. & SUTCLIFFE, D. A new colorimetric reagent for carbohydrates 122

McCARTHY, E. F. & McDOUGALL, E. I. Absorption of immune globulin by the young lamb after ingestion of colostrum 177
McCREA, J. F. Studies on influenza virus receptor-substance and receptor-substance analogues. 1. Preparation and properties of a homogeneous mucoid from the salivary gland of sheep 132
McDOUGALL, E. I. *see* McCARTHY, E. F. 177
McEVOY-BOWE, E. *see* LATNER, A. L. xxiii
McILWAIN, H. Glucose level, metabolism, and response to electrical impulses in cerebral tissues from man and laboratory animals 618
McILWAIN, H. *see also* AYRES, P. J. W. 607
McLEAN, P. *see* GLOCK, G. E. 400
McNAIR SCOTT, D. B. & COHEN, S. S. The oxidative pathway of carbohydrate metabolism in *Escherichia coli*. 1. The isolation and properties of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase 23
McNAIR SCOTT, D. B. & COHEN, S. S. The oxidative pathway of carbohydrate metabolism in *Escherichia coli*. 2. Quantitative studies of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase 33
MACTURK, H. M. *see* FRANCIS, J. 596
MADINAVEITIA, J. *see* FRANCIS, J. 596
MAGUIRE, M. F. *see* LONG, C. xv
MANNERS, D. J. Periodate oxidation of glycogens xx
MAPSON, L. W. The estimation of oxidized glutathione 714
MAPSON, L. W. *see also* CHEN, Y.-T. 821
MARTIN, H. F. & PEERS, F. G. Oat lipase 523
MASSEY, V. Studies on fumarase. 4. The effects of inhibitors on fumarase activity 172
MAW, G. A. Observations on the fate of some aliphatic sulphonic acids in the rat 37
MAW, G. A. The oxidation of dimethylthetin and related compounds to sulphate in the rat 42
MAWSON, C. A. & FISCHER, M. I. Zinc and carbonic anhydrase in human semen 696
MEARA, M. L. *see* CAMA, J. S. 365
MITCHELL, I. L. S. *see* JOHNSTONE, J. H. xvii
MOORE, T. *see* WARD, R. J. 295
MORRIS, C. J. O. R. A fully automatic apparatus for solid-liquid phase countercurrent distribution 369
MORRISON, J. F. Purification and some properties of aconitase iv
MORTON, R. A. *see* LOWE, J. S. 681
MORTON, R. K. Alkaline phosphatase of milk. 1. Association of the enzyme with a particulate lipoprotein complex 786
MORTON, R. K. Alkaline phosphatase of milk. 2. Purification of the enzyme 795
MOSS, J. A. *see* BOWES, J. H. 735
MULDER, H. E. W. *see* MYERS, D. K. i
MUNRO, H. N. *see* WIKRAMANAYAKE, T. W. 640
MYERS, D. K. Studies on cholinesterase. 9. Species variation in the specificity pattern of the pseudo cholinesterases 67
MYERS, D. K., MULDER, H. E. W. & SIMONS, E. G. L. Tocopherol deficiency produced by tri-o-cresyl phosphate i
MYERS, D. K., SIMONS, S. P. & SIMONS, E. G. L. Androgenic hormones and ali-esterase inhibition i

NAISMITH, D. J. *see* WIKRAMANAYAKE, T. W. 640
NASH, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction 416
NEUBERGER, A. *see* ARNSTEIN, H. R. V. 259, 271
NEWTON, B. A. Reversal of the antibacterial activity of polymyxin B by non-toxic divalent cations x
NICHOLAS, R. E. H. & RIMINGTON, C. Studies on the 'Waldenström porphyrin' of acute prophyria urines 109
NIJKAMP, H. J. *see* BROUWER, E. 444
NOBLE, R. L. *see* HULLIN, R. P. 289
NORMAN, J. M. *see* ANSELL, G. B. 768
NORYMBERSKI, J. K. & SERMIN, A. T. Oxidative fission of pregnanediol glucuronide 876
NORYMBERSKI, J. K. *see also* BROOKS, C. J. W. 371

O'DEA, J. F. & GIBBONS, R. A. The estimation of small amounts of formaldehyde liberated during the oxidation of carbohydrates and other substances with periodate 580
OLLEY, J. *see* LOVERN, J. A. 686
ORMEROD, J. G. *see* ELSDEN, S. R. iii

PARKE, D. V. & WILLIAMS, R. T. Studies in detoxication. 54. The metabolism of benzene. (*a*) the formation of phenylglucuronide and phenylsulphuric acid from [^{14}C]benzene. (*b*) The metabolism of [^{14}C]phenol 337
PARKE, D. V. *see also* AZOUZ, W. M. 146
PATEL, M. D. *see* DAGLEY, S. xxxvi
PEARCE, R. H. The turbidimetric estimation of hyaluronate 472
PEARCE, R. H. The turbidimetric estimation of hyaluronidase 467
PEERS, F. G. *see* MARTIN, H. F. 523
PERKINS, D. J. A study of the complexes of some simple peptides with zinc and cadmium ions in aqueous solution xxviii
PERKINS, D. J. A study of the effect of amino acid structure on the stabilities of the complexes formed with metals of group II of the periodic classification 649
PERRY, S. V. The protein components of the isolated myofibril 114
PERRY, S. V. *see also* CHAPPELL, J. B. 586
PIPER, S. H. *see* CHIBNALL, A. C. 707, 711
PODOSKI, E. P. *see* KOGUT, M. 800
POLLARD, C. J. *see* BIERI, J. G. xxxi
PRATT, O. E. The proportion of phosphatase activity demonstrable in brain by histological techniques 140
PREEDY, J. R. K. *see* AITKEN, E. H. 211; HOLIDAY, E. R. 214

RAISTRICK, H., STICKINGS, C. E. & THOMAS, R. Studies in the biochemistry of micro-organisms. 90. Alternariol and alternariol monomethyl ether, metabolic products of *Alternaria tenuis* 421
RAKOVER, D. *see* GUGGENHEIM, K. 388
REES, K. R. Aerobic metabolism of the hepatopancreas of *Helix pomatia* 478
REES, K. R. *see also* JUDAH, J. D. 664
REISS, O. K. & BARRY, J. M. The synthesis of lactose from glucose in the mammary gland 783
RENNIE, S. D. *see* CROMWELL, B. T. 189
RICHTER, D. *see* GAITONDE, M. K. viii
RIMINGTON, C. *see* CANIVET, J. 867; KENNARD, O. 105; NICHOLAS, R. E. H. 109

Rizk, V. *see* Boursnell, J. C. 745
Robertson, H. A. & Barnett, A. J. G. The breakdown of amino acids in fermentation mixtures of minced kale and water xx
Robinson, D., Smith, J. N. & Williams, R. T. Studies in detoxication. 52. The apparent dissociation constants of some glucuronides, mercapturic acids and related compounds 151
Robinson, E. The enzyme complement of bean root cells xxx
Robson, A. *see* Corfield, M. C. 517
Rossiter, R. J. *see* Deluca, H. A. 193; Findlay, M. 200
Rowsell, E. V. Acetylcholine metabolism in electrically stimulated slices of cerebral cortex v
Roy, A. B. The sulphatase of ox liver. 2. The purification and properties of sulphatase A 653
Ruthven, C. R. J. *see* Cole, P. G. 17

Samuels, P. J. The assimilation of amino acids by bacteria. 17. Synthesis of glutathione by extracts of *Escherichia coli* 441
Sermin, A. T. *see* Norymberski, J. K. 876
Shimoni, A. *see* Bergmann, F. 50
Shimoni, E. *see* Bergmann, F. 888
Shorland, F. B. *see* Hansen, R. P. 662
Simons, E. G. L. *see* Myers, D. K. i
Simons, S. P. *see* Myers, D. K. i
Simpson, J. R. & Evans, W. C. The metabolism of nitrophenols by certain bacteria xxiv
Slater, E. C. & Cleland, K. W. The effect of calcium on the respiratory and phosphorylative activities of heart-muscle sarcosomes 566
Slater, E. C. & Holton, F. A. Oxidative phosphorylation coupled with the oxidation of α-ketoglutarate by heart-muscle sarcosomes. 1. Kinetics of the oxidative phosphorylation reaction and adenine nucleotide specificity 530
Slater, E. C. & Lewis, S. E. The effect of dinitrophenol on insect sarcosomes xxvii
Slater, E. C. *see also* Lewis, S. E. xxvii
Smith, J. N. Studies in detoxication. 53. The glucuronic acid conjugation of hydroxyquinolines and hydroxypyridines in the rabbit 156
Smith, J. N. *see also* Friedler, L. vi; Robinson, D. 151
Smith, M. J. H. & Taylor, K. W. The separation of α-keto acids in blood and urine by paper chromatography xxx
Smithies, O. A dynamic osmometer for accurate measurements of small quantities of material: osmotic pressures of isoelectric β-lactoglobulin solutions 57
Smithies, W. R. The lysing action of enzymes on a sample of mycelium of *Penicillium griseofulvum* Dierckx 346
Snow, G. A. *see* Francis, J. 596
Spencer, B. *see* Dodgson, K. S. 253, 315, 436
Srinivasan, M. & Bhatia, I. S. The carbohydrates of *Agave vera cruz* Mill. 286
Stanier, W. M. *see* Consden, R. 248
Stewart, H. B. Isomerism of 2:4-dinitrophenylhydrazones of monobasic α-oxo acids xxvi
Stickings, C. E. *see* Raistrick, H. 421
Stocken, L. A. *see* Ennor, A. H. 310
Stokes, W. M. *see* Klyne, W. xxviii
Stone, B. A. *see* Crook, E. M. xxv
Strickland, K. P. *see* Deluca, H. A. 193; Findlay, M. 200
Sutcliffe, D. *see* Lunt, E. 122
Swain, T. *see* Baruah, P. 392

Taylor, K. W. *see* Smith, M. J. H. xxx
Taylor, W. *see* Brownie, A. C. xxii
Terner, C. Synthesis and oxidation of citric acid in mammary gland homogenates xxix
Thomas, R. *see* Raistrick, H. 421
Thompson, C. C. *see* Barlow, A. J. E. xxxi
Thompson, R. *see* Jackson, H. xxxvi
Todd, A. R. *see* Hackman, R. H. 631

Volcani, B. E. *see* Katchalski, E. 671

Waley, S. G. & Watson, J. The action of trypsin on polylysine 328
Ward, R. J. & Moore, T. 7-Dehydrosterol in the sexual organs of the rat 295
Watson, J. *see* Waley, S. G. 328
Weil-Malherbe, H. The catalytic effect of molybdate on the hydrolysis of organic phosphates 741
Westall, R. G. The amino acids and other ampholytes of urine. 2. The isolation of a new sulphur-containing amino acid from cat urine 244
Wheatley, V. R. Studies of sebum. 4. The estimation of squalene in sebum and sebum-like materials 637
Whittam, R. & Davies, R. E. Active transport of water, sodium, potassium and α-oxoglutarate by kidney-cortex slices 880
Wiggins, H. S. *see* Wootton, I. D. P. 292
Wijesundera, S. & Woods, D. D. The effect of growth on a medium containing methionine on the synthesis of this amino acid by *Bacterium coli* viii
Wikramanayake, T. W., Munro, H. N., Naismith, D. J. & Hutchison, W. C. A note on the influence of energy intake on phospholipid metabolism 640
Williams, E. F. *see* Chibnall, A. C. 707, 711
Williams, R. T. *see* Azouz, W. M. 146; Parke, D. V. 337; Robinson, D. 151
Williamson, D. H. *see* Hughes, D. E. 851
Wiltshire, G. H. The estimation of D- and L-glutamic acid in proteins 46
Wiltshire, G. H. The oxidation of tryptophan in pea-seedling tissues and extracts 408
Winteringham, F. P. W., Loveday, P. M. & Hellyer, G. C. Phosphorus metabolism in the housefly *Musca domestica* xxxiii
Wolff, W. *see* Cuthbertson, W. F. J. 669
Wood, D. R. *see* Kornberg, H. L. ii, iii
Wood, G. C. The stabilization of elastin by a polysaccharide xxxiv
Woods, D. D. *see* Back, K. J. C. xii; Wijesundera, S. viii
Wootton, I. D. P. & Wiggins, H. S. Studies in the bile acids. 2. The non-ketonic acids of human bile 292
Work, E. *see* Lindan, O. 554, 562
Wormall, A. *see* Almond, A. E. xxxiii
Wurzel, M. *see* Bergmann, F. 888
Wyatt, G. R. & Cohen, S. S. The bases of the nucleic acids of some bacterial and animal viruses: the occurrence of 5-hydroxymethylcytosine 774

Yemm, E. W. & Folkes, B. F. The amino acids of cytoplasmic and chloroplastic proteins of barley 700
Young, L. *see* Hawkins, J. B. ix

Index of Subjects

Note. Entries marked with an asterisk (*) refer to communications made in title only.
Entries marked with a section mark (§) refer to contributions to Symposia.
Small roman numerals refer to pages in *Proceedings of the Biochemical Society.*

Acetanilides, substituted, and related compounds, fungicidal activity of (Clark, N. G. & Hams, A. F.) 839
Acetate and glucose, observations on oxidation of, by lactating sheep mammary tissue *in vitro* (Duncombe, W. G. & Glascock, R. F.) xxiii
Acetate, production of keto acids from, by a vibrio (Dagley, S. & Patel, M. D.) xxxvi
3β-Acetoxysteroids, precipitation by digitonin (Haslam, R. M. & Klyne, W.) 340
Acetyl groups, incorporation of ^{14}C from cystine into (Arnstein, H. R. V. & Crawhall, J. C.) 280
Acetylacetone, use in estimation of formaldehyde (Nash, T.) 416
Acetylcholine metabolism in electrically stimulated slices of cerebral cortex (Rowsell, E. V.) v
Acid anhydrides, enzymic hydrolysis of (Bergmann, F., Wurzel, M. & Shimoni, E.) 888
Acids, bile, studies in (Wootton, I. D. P. & Wiggins, H. S.) 292
Acids, component, of milk fats of *Balaenoptera physalus* (finner whale) (Cama, J. S. & Meara, M. L.) 365
Acids, fatty *see* Fatty acids
Acids, phenolic, excretion in urines of rat (Boscott, R. J. & Greenberg, S. M.) xviii
Aconitase in *Pseudomonas fluorescens* (Kogut, M. & Podoski, E. P.) 800
Aconitase, purification and properties (Morrison, J. F.) iv
*cis*Aconitate in pigeon brain (Coxon, R. V.) 545
Aconitic acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Adaptation, simultaneous (Kogut, M. & Podoski, E. P.) 800
Adenine nucleotide specificity, kinetics of (Slater, E. C. & Holton, F. A.) 530
Adenosine diphosphate and oxidative phosphorylation in heart-muscle sarcosomes (Slater, E. C. & Holton, F. A.) 530
Adenosine diphosphate in muscle of tortoise (Caldwell, P. C.) 458
Adenosine diphosphate, protection of α-ketoglutaric oxidase and succinic oxidase by (Slater, E. C. & Cleland, K. W.) 566
Adenosine monophosphate and oxidative phosphorylation in heart-muscle sarcosomes (Slater, E. C. & Holton, F. A.) 530
Adenosine triphosphatase activities of skeletal-muscle mitochondria (Chappell, J. B. & Perry, S. V.) 586
Adenosine triphosphatase activity in intestinal mucosa (Hele, M. P.) 857
Adenosine triphosphatase in brain (Gordon, J. J.) 812; (Pratt, O. E.) 140
Adenosine triphosphate and oxidative phosphorylation in heart-muscle sarcosomes (Slater, E. C. & Holton, F. A.) 530
Adenosine triphosphate as a primer for fatty acid oxidation (Judah, J. D. & Rees, K. R.) 664
Adenosine triphosphate as competitive inhibitor of 6-phosphogluconate dehydrogenase (Glock, G. E. & McLean, P.) 400
Adenosine triphosphate, concentration in rat muscle, and effects of cortisone, deoxycorticosterone and adrenalectomy (Conway, E. J. & Hingerty, D.) 447
Adenosine triphosphate in muscle of tortoise (Caldwell, P. C.) 458
Adenosine triphosphate in oxidation of glycerol by mycobacteria (Hunter, G. J. E.) 320
Adenosine triphosphate, ^{32}P-labelled, preparation of (Hems, R. & Bartley, W.) 434
Adipic acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Adrenal steroids, effect on active transport of sodium and potassium ions in yeast (Conway, E. J. & Hingerty, D.) 455
Adrenal steroids, effect on aspects of organic and inorganic metabolism (Conway, E. J. & Hingerty, D.) 447
Adrenalectomized rats, deoxycorticosterone acetate and cortisone on response of, to hypotonic load (Cole, D. F.) xiii
Aerobacter aerogenes, dissimilation of citric acid by (Dagley, S. & Dawes, E. A.) xvi
Affinities of enzymes for substrates and inhibitors, effect of pH (Dixon, M.) 162
Agave vera cruz Mill., carbohydrates of (Srinivasan, M. & Bhatia, I. S.) 286
Agglutination of bacteria by polyamino acids (Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E.) 671
Alanine, incorporation of ^{14}C from cystine into (Arnstein, H. R. V. & Crawhall, J. C.) 280
Alanine, *N*-benzoyl derivative, chromatography of (Arnstein, H. R. V. & Neuberger, A.) 271
Ali-esterase inhibition, and androgenic hormones (Myers, D. K., Simons, S. P. & Simons, E. G. L.) i
Alkali-treated proteins, hydrolysates of, optical form of glutamic acid in (Wiltshire, G. H.) 46
Alternaria tenuis, alternariol and derivatives, metabolic products from (Raistrick, H., Stickings, C. E. & Thomas, R.) 421
Alternariol and derivatives, metabolic products of *Alternaria tenuis* (Raistrick, H., Stickings, C. E. & Thomas, R.) 421
Amaranthus caudatus L. leaves, glycinebetaine content of (Cromwell, B. T. & Rennie, S. D.) 189
Amines, reaction of catechol derivatives with, in presence of oxidizing agents (Hackman, R. H. & Todd, A. R.) 631
Amino acid incorporation by fragmented staphylococci (Gale, E. F. & Folkes, J. P.) xi
Amino acid structure, effect on stabilities of complexes formed with metals of group II of periodic classification (Perkins, D. J.) 649
Amino acid, sulphur-containing, isolation from cat urine (Westall, R. G.) 244
Amino acids and other ampholytes of urine (Westall, R. G.) 244
Amino acids, assimilation by bacteria (Gale, E. F. & Folkes, J. P.) 721, 730; (Samuels, P. J.) 441
Amino acids, breakdown in fermentation mixtures of minced kale and water (Robertson, H. A. & Barnett, A. J. G.) xx
Amino acids, determination by ^{64}Cu in salmine (Corfield, M. C. & Robson, A.) 517
Amino acids from mucoprotein of sheep (McCrea, J. F.) 132

Amino acids, microbiological assay of (Yemm, E. W. & Folkes, B. F.) 700
Amino acids of cytoplasmic and chloroplastic proteins of barley (Yemm, E. W. & Folkes, B. F.) 700
Amino acids, reaction of catechol derivatives with (Hackman, R. H. & Todd, A. R.) 631
Amino acids, unsaturated, colour reaction with ninhydrin (Fowden, L. & Done, J.) 548
α-Amino acids, water-soluble, action on bacteria (Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E.) 671
α-Aminoethanesulphonic acid, stability in aqueous solution (Maw, G. A.) 37
Aminomethanesulphonic acid, stability in aqueous solution (Maw, G. A.) 37
Amino groups, reaction with fluorodinitrobenzene (Bowes, J. H. & Moss, J. A.) 735
γ-Amino-α-methylenebutyric acid, occurrence in groundnut plants (Fowden, L. & Done, J.) 548
Aminophenylglucuronides, absorption spectra and optical rotations (Robinson, D., Smith, J. N. & Williams, R. T.) 151
Aminopterin, effect on excretion in urines of phenolic acids in rat (Boscott, R. J. & Greenberg, S. M.) xviii
Ammonium ions and biosynthesis of choline by *Cicer arietinum* (Ahmad, K. & Karim, M. A.) 817
Ampholytes of urine (Westall, R. G.) 244
*iso*Amyl alcohol, cysteine derivative in cat urine (Westall, R. G.) 244
Androgenic hormones and ali-esterase inhibition (Myers, D. K., Simons, S. P. & Simons, E. G. L.) i
Aneurin pyrophosphatase in brain (Pratt, O. E.) 140
Anhydrides, acid, enzymic hydrolysis of (Bergmann, F., Wurzel, M. & Shimoni, E.) 888
Antibacterial activity of polymyxin B, reversal by non-toxic divalent cations (Newton, B. A.) x
Antibiotics, effect on excretion of phenolic acids in rat urines (Boscott, R. J. & Greenberg, S. M.) xviii
Antibiotics, inhibition protein synthesis by (Gale, E. F. & Folkes, J. P.) 721
Antibiotics, lack of effect on glutathione synthesis by extracts of *Escherichia coli* (Samuels, P. J.) 441
Antigens, ^{131}I-labelled, fate in immune and normal rabbits (Almond, A. E., Francis, G. E., Hawkins, J. D. & Wormall, A.) xxxiii
Antigens, red-cell, marked with ^{131}iodine (Boursnell, J. C., Coombs, R. R. A. & Rizk, V.) 745
Antihaemagglutinin, mucoid, from sheep (McCrea, J. F.) 132
Antisera, marked, studies with (Boursnell, J. C., Coombs, R. R. A. & Rizk, V.) 745
Anti-thrombic action of heparin, effect of reducing agents on (Green, J.) xxiii
Araboascorbic acid, estimation by chromatography (Chen, Y.-T., Isherwood, F. A. & Mapson, L. W.) 821
Arachis hypogaea, γ-amino-α-methylenebutyric acid in (Fowden, L. & Done, J.) 548
Arginase, effect of pH on Michaelis constants (Dixon, M.) 162
Arsenite inhibition of oxidation of glycerol by mycobacteria (Hunter, G. J. E.) 320
Arylsulphatase of limpets, preparation free from β-glucuronidase (Dodgson, K. S. & Spencer, B.) 315
Arylsulphatase of marine molluscs (Dodgson, K. S., Lewis, J. I. M. & Spencer, B.) 253
Arylsulphuric acids, ^{35}S-labelled, fate after administration to rat (Hawkins, J. B. & Young, L.) ix
Ascorbic acid and *iso*ascorbic acid, detoxifying effect on cyanide, phenol and toxin (Guggenheim, K., Leibowitz, J. & Rakover, D.) 388
D-*iso*Ascorbic acid, chemical properties and biological activities of (Guggenheim, K., Leibowitz, J. & Rakover, D.) 388
L-Ascorbic acid, chemical properties and biological activities of (Guggenheim, K., Leibowitz, J. & Rakover, D.) 388
Ascorbic acid, effect on excretion of phenolic acids in rat urines (Boscott, R. J. & Greenberg, S. M.) xviii
L-Ascorbic acid, effect on *in vitro* activity of polyphenoloxidase from potato (Baruah, P. & Swain, T.) 392
Ascorbic acid, estimation by chromatography (Chen, Y.-T., Isherwood, F. A. & Mapson, L. W.) 821
Ascorbic acid level, changes in dietetic liver necrosis (Lindan, O. & Work, E.) 554
Ascorbic acid, level in liver subjected to acute circulatory congestion in relation to level in dietetic liver necrosis (Lindan, O. & Work, E.) 562
Ash in fractions of bacterial cells (Fernell, W. R. & King, H. K.) 758
Aspartic acid, incorporation of ^{14}C from cystine into (Arnstein, H. R. V. & Crawhall, J. C.) 280
Aspergillus flavus, action on oligosaccharides (Bealing, F. J.) 93
Aspergillus niger, action on oligosaccharides (Bealing, F. J.) 93
Aspergillus oryzae, action on oligosaccharides (Bealing, F. J.) 93
Asymmetry of purified sheep salivary mucoid (Caspary, E. A.) 139
Atriplex canescenes and related plants, glycinebetaine content of (Cromwell, B. T. & Rennie, S. D.) 189

Bacteria, action of water-soluble poly-α-amino acids on (Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E.) 671
Bacteria, assimilation of amino acids by (Gale, E. F. & Folkes, J. P.) 721, 730; (Samuels, P. J.) 441
Bacteria, deamidation of nicotinamide by (Hughes, D. E. & Williamson, D. H.) 851
Bacteria, metabolism of nitrophenols by (Simpson, J. R. & Evans, W. C.) xxiv
Bacteria, soluble and insoluble fractions of (Fernell, W. R. & King, H. K.) 758
Bacteriophages, 5-hydroxymethylcytosine in deoxyribonucleic acid of (Wyatt, G. R. & Cohen, S. S.) 774
Bacterium coli, effect of growth on medium containing methionine on synthesis by (Wijesundera, S. & Woods, D. D.) viii
Bacterium coli, requiring citrulline and a pyrimidine for growth (Back, K. J. C. & Woods, D. D.) xii
Balaenoptera physalus (finner whale), milk fats of (Cama, J. S. & Meara, M. L.) 365
Barley, cytoplasmic and chloroplastic proteins of, amino acids of (Yemm, E. W. & Folkes, B. F.) 700
Barley, decarboxylase for γ-methyleneglutamic acid (Fowden, L. & Done, J.) 548
Bean root cells, enzymes of (Robinson, E.) xxx
Benzene, metabolism of (Parke, D. V. & Williams, R. T.) 337
Beta vulgaris L., estimation and distribution of glycinebetaine in (Cromwell, B. T. & Rennie, S. D.) 189
Betaines in plants, biosynthesis and metabolism (Cromwell, B. T. & Rennie, S. D.) 189
Bile acids, studies in (Wootton, I. D. P. & Wiggins, H. S.) 292
Bile, diazo reacting pigments of (Cole, P. G., Lathe, G. H. & Billing, B. H.) xiii
Bismuthate, oxidation of pregnanediol glucuronide with (Norymberski, J. K. & Sermin, A. T.) 876
Bismuthate, sodium, oxidation of corticosteroids with (Brooks, C. J. W. & Norymberski, J. K.) 371
Blood glutathione level, changes in dietetic liver necrosis (Lindan, O. & Work, E.) 554
Blood, separation of α-keto acids in, by paper chromatography (Smith, M. J. H. & Taylor, K. W.) xxx

Brain dispersion, lipids of, incorporation of labelled phosphate into (Dawson, R. M. C.) 507
Brain, phosphatases of (Gordon, J. J.) 812
Brain, proportion of phosphatase activity demonstrable by histological techniques in (Pratt, O. E.) 140
Brain, pyruvate oxidation by (Coxon, R. V.) 545
Brain, rat, glycerylphosphorylethanolamine in (Ansell, G. B. & Norman, J. M.) 768
Brain, rat, protein synthesis in (Gaitonde, M. K. & Richter, D.) viii
Brain slices, nucleic acids of, incorporation of radioactive phosphate into (Deluca, H. A., Rossiter, R. J. & Strickland, K. P.) 193
Brain slices, pentosenucleic acids of, factors affecting incorporation of radioactive phosphate into (Findlay, M., Rossiter, R. J. & Strickland, K. P.) 200
Bulls, urine, lipids from (Broadbent, I. E., Brooks, R. V. & Klyne, W.) vi
Butterfat, branched-chain fatty acids of (Hansen, R. P. & Shorland, F. B.) 662
n-Butyrate, formed from lower fatty acids by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183

Cadmium ions in aqueous solution, complexes of simple peptides with (Perkins, D. J.) xxviii
Calcium, effect on respiration and phosphorylation in heart-muscle sarcosomes (Salter, E. C. & Cleland, K. W.) 566
Capsicum, decarboxylase for γ-methyleneglutamic acid (Fowden, L. & Done, J.) 548
Carbohydrate metabolism, oxidative pathway in *Escherichia coli* (McNair Scott, D. B. & Cohen, S. S.) 23, 33
Carbohydrates, colorimetric determination of (Lunt, E. & Sutcliffe, D.) 122
Carbohydrates of *Agave vera cruz* Mill. (Srinivasan, M. & Bhatia, I. S.) 286
Carbohydrates, periodate oxidation of, estimation of formaldehyde liberated during (O'Dea, J. F. & Gibbons, R. A.) 580
Carbohydrates, spectroscopic studies (Love, R. M.) 126
Carbon dioxide, formation by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Carbonic acid, dehydration, kinetics (Dalziel, K.) 79
Carbonic anhydrase in human semen, spermatozoa and seminal plasma (Mawson, C. A. & Fischer, M. I.) 696
Carcinogens, role of soluble proteins in elimination of (Chalmers, J. G.) xix
Carotene, site of conversion of, in rats (Bieri, J. G. & Pollard, C. J.) xxxi
Carotenoids in larvae of *Plusia gamma* (Goodwin, T. W.) 834
Cat urine, isolation of new sulphur-containing amino acid from (Westall, R. G.) 244
Catechol derivatives, oxidation in presence of amines and amino acids (Hackman, R. H. & Todd, A. R.) 631
Catechols, colorimetric determination of (Azouz, W. M., Parke, D. V. & Williams, R. T.) 146
Catechols, formation of, from halogenobenzenes in rabbit (Azouz, W. M., Parke, D. V. & Williams, R. T.) 146
Cations, divalent non-toxic, reversal of antibacterial activity of polymyxin B by (Newton, B. A.) x
Cerebral cortex, electrically stimulated, acetylcholine metabolism in (Rowsell, E. V.) v
Cerebral cortex, electrically stimulated, effect of metabolic inhibitors on respiration and glycolysis in (Heald, P. J.) 625
Cerebral tissues, glucose level, metabolism, and response to electrical impulses in (Mellwain, H.) 618
Cerebrin, yeast, α-hydroxy-*n*-fatty acids of (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 711
Cerebronic acid, observations on (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 707
Cerebrospinal fluid proteins, separation by paper electrophoresis (Eaton, J. C. & Gardner, M. D.) xxv
Cerebrum phospholipids of, complete fractionation (Dawson, R. M. C.) xii
Chenodeoxycholic acid, preparation of (Wootton, I. D. P. & Wiggins, H. S.) 292
Chenopodium leaves, glycinebetaine content of (Cromwell, B. T. & Rennie, S. D.) 189
Chick-pea seedlings, biosynthesis of choline in (Ahmad, K. & Karim, M. A.) 817
Chitin, of fungal mycelium, action of enzymes on (Smithies, W. R.) 346
Chloramphenicol, inhibition protein synthesis in *Staphylococcus aureus* by (Gale, E. F. & Folkes, J. P.) 730
Chloretone, effect on excretion of phenolic acids in rat urines (Boscott, R. J. & Greenberg, S. M.) xviii
p-Chlorophenylalanine, inhibition protein synthesis in *Staphylococcus aureus* by (Gale, E. F. & Folkes, J. P.) 730
Chloroplastic proteins of barley, amino acids of (Yemm, E. W. & Folkes, B. F.) 700
Cholesterol, free and combined, in plasma (Kerr, L. M. H. & Bauld, W. S.) 872
Cholesterol, possible role of coenzyme A in biosynthesis of (Boyd, G. S.) 892
Choline, biosynthesis in seedling of chick-pea (Ahmad, K. & Karim, M. A.) 817
Choline, synthesis of, effect of cobalamin on quantitative utilization of serine, glycine and formate for (Arnstein, H. R. V. & Neuberger, A.) 259
Choline of placenta (Cole, P. G., Lathe, G. H. & Ruthven, C. R. J.) 17
Cholinesterase, effect of alkyl fluoroacetates (Bergmann, F. & Shimoni, A.) 50
Cholinesterase, effect of pH on Michaelis constants (Dixon, M.) 162
Cholinesterase, studies on (Myers, D. K.) 67
Cholinesterases, mechanism of inhibition by organophosphorus compounds (Aldridge, W. N. & Davison, A. N.) 763
Chromatography of amino acids on starch (Corfield, M. C. & Robson, A.) 517
Chromatography, paper, separation of phosphate esters of muscle by (Caldwell, P. C.) 458
Chromatography, paper, separation of uroporphyrin esters I and III by (Falk, J. E. & Benson, A.) 101
Cicer arietinum, biosynthesis of choline in (Ahmad, K. & Karim, M. A.) 817
Citrate in pigeon brain (Coxon, R. V.) 545
*iso*Citrate in pigeon brain (Coxon, R. V.) 545
Citrate oxidized by mitochondria (Chappell, J. B. & Perry, S. V.) 586
Citric acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Citric acid, dissimilation by *Aerobacter aerogenes* (Dagley, S. & Dawes, E. A.) xvi
Citric acid, enzymic estimation of (Dagley, S. & Dawes, E. A.) xxxv
Citric acid in mammary gland homogenates, synthesis and oxidation of (Terner, C.) xxix
*iso*Citric dehydrogenase in *Pseudomonas fluorescens* (Kogut, M. & Podoski, E. P.) 800
Citrulline, anaerobic breakdown and phosphorylation (Knivett, V. A.) x
Citrulline and a pyrimidine, required for growth of a strain of *Bacterium coli* (Back, K. J. C. & Woods, D. D.) xii
Cobalamin, effect on quantitative utilization of serine, glycine and formate for synthesis of choline and methyl groups of methionine (Arnstein, H. R. V. & Neuberger, A.) 259
Cobalamin, influence on growth on diets deficient in labile methyl groups in rats (Arnstein, H. R. V. & Neuberger, A.) 259

Cobalt and iron, mechanism of effect of, on production of toxin by *Corynebacterium diphtheriae* (Clarke, W. A. & Clarke, G. D.) xvi
Coccus, Gram-negative, production of fatty acids by (Elsden, S. R. & Lewis, D.) 183
Coenzyme A, possible role in biosynthesis of cholesterol in rat (Boyd, G. S.) 892
Collagen from rheumatic fever patients (Consden, R., Glynn, L. E. & Stanier, W. M.) 248
Collagen, reaction with fluorodinitrobenzene (Bowes, J. H. & Moss, J. A.) 735
Collodion membranes, preparation of (Smithies, O.) 57
Colostrum, immune globulin in young lamb (McCarthy, E. F. & McDougall, E. I.) 177
Complexes formed with metals of group II of periodic classification, effect of amino acid structure on stabilities of (Perkins, D. J.) 649
Connective tissue, chemical examination in rheumatic fever (Consden, R., Glynn, L. E. & Stanier, W. M.) 248
Copper in hepatic cells, effect of fasting (Harrison, M. F.) 204
Copper ions, effect on polyphenoloxidase (Baruah, P. & Swain, T.) 392
Copper, radioactive, use in determining amino acids (Corfield, M. C. & Robson, A.) 517
Coproporphyrins I and III, paper chromatography of (Falk, J. E. & Benson, A.) 101
Corticosteroids, oxidation with sodium bismuthate (Brooks, C. J. W. & Norymberski, J. K.) 371
Cortisone and deoxycorticosterone acetate on response of adrenalectomized rats to hypotonic load (Cole, D. F.) xiii
Cortisone and deoxycorticosterone effects on aspects of organic and inorganic metabolism (Conway, E. J. & Hingerty, D.) 447
Cortisone, effect on active transport of sodium and potassium ions in yeast (Conway, E. J. & Hingerty, D.) 455
Corynebacterium diphtheriae, production of toxin by, mechanism of effect of iron and cobalt on (Clarke, W. A. & Clarke, G. D.) xvi
Countercurrent distribution, solid-liquid phase, fully automatic apparatus for (Morris, C. J. O. R.) 369
Countercurrent methods, application to fractionation of lipid material from human placenta (Cole, P. G., Lathe, G. H. & Ruthven, C. R. J.) 17
Cows, pregnant urine, lipids from (Broadbent, I. E., Brooks, R. V. & Klyne, W.) vi
Creatine phosphate in muscle of tortoise (Caldwell, P. C.) 458
Creatine in urine, application of diacetyl reaction to estimation of (Ennor, A. H. & Stocken, L. A.) 310
Creatinine and biosynthesis of choline by *Cicer arietinum* (Ahmad, K. & Karim, M. A.) 817
Cutaneous porphyria, uroporphyrins of (Nicholas, R. E. H. & Rimington, C.) 109
Cyanide, complexes with ferriperoxidase (George, P.) 220
Cyanide, effect on incorporation of phosphate into nucleic acids (Deluca, H. A., Rossiter, R. J. & Strickland, K. P.) 193
Cyanide inhibition of oxidation of glycerol by mycobacteria (Hunter, G. J. E.) 320
Cysteine, *S*-hydroxyalkyl derivative in cat urine (Westall, R. G.) 244
DL-Cysteinol and derivatives, preparation and properties (Crawhall, J. C. & Elliott, D. F.) vii
DL-[β-^{14}C]Cystine, incorporation into alanine, aspartic acid, glutamic acid and acetyl groups (Arnstein, H. R. V. & Crawhall, J. C.) 280
DL-[β-^{14}C]Cystine, synthesis from [^{14}C]methanol (Arnstein, H. R. V. & Crawhall, J. C.) 280
Cytochrome oxidase in muscle; activity in relation to myoglobin content, activity in relation to age of animals (Lawrie, R. A.) 298
Cytochrome-oxidase activity, relation of energy-rich phosphate in muscle to (Lawrie, R. A.) 305
Cytochrome *c* peroxidase and horseradish peroxidase, chemical nature of second hydrogen peroxide compound formed by (George, P.) 220
Cytoplasmic proteins of barley, amino acids of (Yemm, E. W. & Folkes, B. F.) 700

Deamidation of nicotinamide by bacteria (Hughes, D. E. & Williamson, D. H.) 851
Dehydrogenases of *Escherichia coli* (McNair Scott, D. B. & Cohen, S. S.) 23, 33
7-Dehydrosterol in sexual organs of rat (Ward, R. J. & Moore, T.) 295
Deoxycorticosterone acetate and cortisone on response of adrenalectomized rats to hypotonic load (Cole, D. F.) xiii
Deoxycorticosterone and cortisone effects on aspects of organic and inorganic metabolism (Conway, E. J. & Hingerty, D.) 447
11-Deoxycorticosterone (DOC), enzymic 11-β-hydroxylation of (Brownie, A. C., Grant, J. K. & Taylor, W.) xxii
Deoxycorticosterone, effect on active transport of sodium and potassium ions in yeast (Conway, E. J. & Hingerty, D.) 455
Deoxypentose in fractions of bacterial cells (Fernell, W. R. & King, H. K.) 758
Deoxyribonucleic acids, 5-hydroxymethylcytosine in bases of (Wyatt, G. R. & Cohen, S. S.) 774
Detoxication, studies in (Azouz, W. M., Parke, D. V. & Williams, R. T.) 146; (Parke, D. V. & Williams, R. T.) 337; (Robinson, D., Smith, J. N. & Williams, R. T.) 151; (Smith, J. N.) 156
Diacetyl reaction, application to estimation of creatine in urine (Ennor, A. H. & Stocken, L. A.) 310
Diazo reacting pigments of serum, urine and bile (Cole, P. G., Lathe, G. H. & Billing, B. H.) xiii
Dibenzo-α-pyrones in *Alternaria tenuis* (Raistrick, H., Stickings, C. E. & Thomas, R.) 421
Dicarboxylic acids, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Dietary deficiencies, effect on excretion in urines of phenolic acids in rat (Boscott, R. J. & Greenberg, S. M.) xviii
Digitonin, precipitation of steroids by (Haslam, R. M. & Klyne, W.) 340
Dihydroxylation *in vivo* (Azouz, W. M., Parke, D. V. & Williams, R. T.) 146
N-(3:4-Dihydroxyphenyl)aminoacetate, ethyl, synthesis of (Hackman, R. H. & Todd, A. R.) 631
3:5-Diiodotyrosine, intravenously injected, fate in immune and normal rabbits (Almond, A. E., Francis, G. E., Hawkins, J. D. & Wormall, A.) xxxiii
Dilysine, an end product of enzymic hydrolysis (Waley, S. G. & Watson, J.) 328
Dimethylthetin and related compounds, oxidation to sulphate in rat (Maw, G. A.) 42
Dinitrophenol, effect on incorporation of ^{32}P into pentosenucleic acids of brain slices (Findlay, M., Rossiter, R. J. & Strickland, K. P.) 200
Dinitrophenol, effect on insect sarcosomes (Slater, E. C. & Lewis, S. E.) xxvii
2:4-Dinitrophenol inhibition of fatty acid oxidation (Judah, J. D. & Rees, K. R.) 644
2:4-Dinitrophenylhydrazones of monobasic α-oxo acids, isomerism of (Stewart, H. B.) xxvi
Dissociation constants of glucuronides, mercapturic acids and hippuric acids (Robinson, D., Smith, J. N. & Williams, R. T.) 151
Dog, hair grease of, occurrence of two valeric acids, α- and β-methylbutyric acids in (Brouwer, E. & Nijkamp, H. J.) 444

Elastin from rheumatic fever patients (Consden, R., Glynn, L. E. & Stanier, W. M.) 248

Elastin, physical studies of degradation product from (Bowen, T. J.) 766
Elastin, stabilization by a polysaccharide (Wood, G. C.) xxxiv
Elastin-elastase system, studies on complex nature of (Hall, D. A.) xxxv
Electrical impulses, application to separated tissues in aqueous media (Ayres, P. J. W. & McIlwain, H.) 607
Electrical impulses, response in cerebral tissues (McIlwain, H.) 618
Electrical stimulation and acetylcholine metabolism (Rowsell, E. V.) v
Electrophoresis, continuous on paper (Holdsworth, E. S.) xiv
Electrophoresis, filter-paper, of mould glycosidases (Bealing, F. J.) 93
Electrophoresis, paper, separation of cerebrospinal fluid proteins by (Eaton, J. C. & Gardner, M. D.) xxv
Electrophoretic mobilities of bacteria (Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E.) 671
Enediols, estimation by chromatography (Chen, Y.-T., Isherwood, F. A. & Mapson, L. W.) 821
Energy intake, influence on phospholipid metabolism (Wikramanayake, T. W., Munro, H. N., Naismith, D. J. & Hutchison, W. C.) 640
Enzyme formation in *Saccharomyces fragilis* (Davies, R.) 484
Enzyme inhibitor constants, determination (Dixon, M.) 170
Enzymes, mechanism of inhibition (Aldridge, W. N. & Davison, A. N.) 763
Enzymes of bean root cells (Robinson, E.) xxx
Escherichia coli, and phages of, nucleic-acid bases (Wyatt, G. R. & Cohen, S. S.) 774
Escherichia coli, glutathione synthesis by (Samuels, P. J.) 441
Escherichia coli, inhibition of, by polyamino acids (Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E.) 671
Escherichia coli, oxidative pathway of carbohydrate metabolism in (McNair Scott, D. B & Cohen, S. S.) 23, 33
Esterases and hydrolysis of alkyl fluoroacetate (Bergmann, F. & Shimoni, A.) 50
Esterases, mechanism of action (Bergmann, F., Wurzel, M. & Shimoni, E.) 888
Ethanolamine of placenta (Cole, P. G., Lathe, G. H. & Ruthven, C. R. J.) 17
Ethylenediaminetetraacetic acid, effect on phosphorylation in mitochondria (Chappell, J. B. & Perry, S. V.) 586
Ethylenediaminetetraacetic acid, protector of α-ketoglutaric oxidase and succinic oxidase (Slater, E. C. & Cleland, K. W.) 566
Exocuticle, insect, hardening of (Hackman, R. H. & Todd, A. R.) 631

Fats, milk, of *Balaenoptera physalus* (finner whale), component acids of (Cama, J. S. & Meara, M. L.) 365
Fatty acid oxidation, activation by kidney and liver mitochondria (Judah, J. D. & Rees, K. R.) 664
Fatty acids, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Fatty acids, α-hydroxy-*n*-, of yeast cerebrin (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 711
Fatty acids, of butterfat (Hansen, R. P. & Shorland, F. B.) 662
Fatty acids, production by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Fatty acids, volatile, formation in laboratory mixtures of minced crops and water, and occurrence in field silage (Barnett, A. J. G. & Duncan, R. E. B.) xix
Felinine from cat urine (Westall, R. G.) 244
Fermentation in mixtures of minced kale and water, breakdown of amino acids (Robertson, H. A. & Barnett, A. J. G.) xx
Fermentation, yeast, nature of inhibition by D-glucosone (Johnstone, J. H. & Mitchell, I. L. S.) xvii
Ferriperoxidase, dissociation constants of (George, P). 220
Fibrin in nodules from rheumatic fever patients (Consden, R., Glynne, L. E. & Stanier, W. M.) 248
Fibrinolysins of human plasma (Bidwell, E.) 497
Fish, lipids of (Lovern, J. A. & Olley, J.) 686
Fish skeletal muscle, proteins of (Connell, J. J.) 378
Flame photometer, direct-reading, precision of, for determination of sodium and potassium in biological fluids (Holiday, E. R. & Preedy, J. R. K.) 214
Fluoride, complexes with ferriperoxidase (George, P.) 220
Fluoride inhibition of oxidation of glycerol by mycobacteria (Hunter, G. J. E.) 320
Fluorine compounds and metabolism of *Rhodospirillum rubrum* (Elsden, S. R. & Ormerod, J. G.) iii
Fluoroacetate inhibition of fatty acid oxidation (Judah, J. D. & Rees, K. R.) 664
Fluoroacetates, alkyl, and related compounds, enzymic hydrolysis of (Bergmann, F. & Shimoni, A.) 50
Folic acid, conversion to citrovorum factor (Guggenheim, K., Leibowitz, J. & Rakover, D.) 388
Formaldehyde, colorimetric estimation by Hantzsch reaction (Nash, T.) 416
Formaldehyde liberated during periodate oxidation, microestimation of (O'Dea, J. F. & Gibbons, R. A.) 580
Formate, metabolism by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Formate, quantitative utilization of, for synthesis of choline and methyl groups of methionine (Arnstein, H. R. V. & Neuberger, A.) 259
Fructose from *Agave vera cruz* Mill. (Srinivasan, M. & Bhatia, I. S.) 286
Fructose, metabolism by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Fructosidase: mould 'glucosaccharase' (Bealing, F. J.) 93
Fucose in sheep salivary mucoid (McCrea, J. F.) 132
Fumarase activity, effects of inhibitors (Massey, V.) 172
Fumarase, effect of pH on Michaelis constants (Dixon, M.) 162
Fumarase in *Pseudomonas fluorescens* (Kogut, M. & Podoski, E. P.) 800
Fumarase, studies (Massey, V.) 172
Fumarate oxidized by mitochondria (Chappell, J. B. & Perry, S. V.) 586
Fumaric acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Fungicidal activity of substituted acetanilides and related compounds (Clark, N. G. & Hams, A. F.) 839
Furan derivatives from sugars (Love, R. M.) 126

Galactose, detection in urine (Fowweather, F. S.) 718
Galactose in nodules from rheumatic fever patients (Consden, R., Glynn, L. E. & Stanier, W. M.) 248
Gastric secretion of cats and urea metabolism (Kornberg, H. L., Davies, R. E. & Wood, D. R.) ii, iii
Gelatin, reaction with fluorodinitrobenzene (Bowes, J. H. & Moss, J. A.) 735
Globulin, immune, absorption by young lamb after ingestion of colostrum (McCarthy, E. F. & McDougall, E. I.) 177
γ-Globulin, serum iodine uptake by (Boursnell, J. C., Coombs, R. R. A. & Rizk, V.) 745
Glucono-1:5-lactone, inhibition of rumen β-glucosidase activity by (Conchie, J.) xxi
'Glucosaccharase', mould: a fructosidase (Bealing, F. J.) 93
Glucosamine in nodules from rheumatic fever patients (Consden, R., Glynn, L. E. & Stanier, W. M.) 248
Glucose and acetate, observations on oxidation of, by lactating sheep mammary tissue *in vitro* (Duncombe, W. G. & Glascock, R. F.) xxiii

Glucose from *Agave vera cruz* Mill. (Srinivasan, M. & Bhatia, I. S.) 286
Glucose in mammary gland, synthesis of lactose from (Reiss, O. K. & Barry, J. M.) 783
Glucose level, metabolism, and response to electrical impulses in cerebral tissues (McIlwain, H.) 618
Glucose, metabolism by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Glucose 6-phosphate dehydrogenase, isolation and properties (McNair Scott, D. B. & Cohen, S. S.) 23
Glucose 6-phosphate dehydrogenase of rat liver, properties and assay (Glock, G. E. & McLean, P.) 400
Glucose 6-phosphate dehydrogenase, quantitative studies (McNair Scott, D. B. & Cohen, S. S.) 33
Glucose, polymers of (Kent, P. W.) 361
β-Glucosidase activity, rumen, inhibition by gluconolactone (Conchie, J.) xxi
β-Glucosides, enzymic hydrolysis of, formation of oligosaccharides during (Crook, E. M. & Stone, B. A.) xxv
D-Glucosone, nature of inhibition of yeast fermentation by (Johnstone, J. H. & Mitchell, I. L. S.) xvii
Glucuronic acid conjugation of hydroxyquinolines and hydroxypyridines in rabbit (Smith, J. N.) 156
β-Glucuronidase of limpets, inhibition of arylsulphatase activity of preparations of (Dodgson, K. S. & Spencer, B.) 315
β-Glucuronidase of marine molluscs (Dodgson, K. S., Lewis, J. I. M. & Spencer, B.) 253
Glucuronide of pregnanediol, oxidative fission of, with sodium bismuthate (Norymberski, J. K. & Sermin, A. T.) 876
Glucuronides, apparent dissociation constants (Robinson, D., Smith, J. N. & Williams, R. T.) 151
Glutamic acid, D-, and L-, estimation in proteins (Wiltshire, G. H.) 46
Glutamic acid, incorporation into protein fraction of *Staphylococcus aureus* (Gale, E. F. & Folkes, J. P.) 721
Glutamic acid, incorporation of ^{14}C from cystine into (Arnstein, H. R. V. & Crawhall, J. C.) 280
Glutamic acid of placenta (Cole, P. G., Lathe, G. H. & Ruthven, C. R. J.) 17
Glutamic acid, optical form of, in hydrolysates of tobacco-mosaic-virus-protein, *Lactobacillus casei*, proteins and alkali-treated proteins (Wiltshire, G. H.) 46
Glutamic acid, racemization of (Wiltshire, G. H.) 46
Glutaric acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Glutathione, level in liver subjected to acute circulatory congestion and in dietetic liver necrosis (Lindan, O. & Work, E.) 562
Glutathione levels, liver blood and spleen, changes in dietetic liver necrosis (Lindan, O. & Work, E.) 554
Glutathione, oxidized, estimation of (Mapson, L. W.) 714
Glutathione, synthesis by bacterial extracts (Samuels, P. J.) 441
Glyceric acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Glycerol, oxidation by mycobacteria (Hunter, C. J. E.) 320
Glycerylphosphorylethanolamine in rat brain (Ansell, G. B. & Norman, J. M.) 768
Glycine, α-benzamido acids, chromatography of; *N*-benzoyl derivatives, chromatography of (Arnstein, H. R. V. & Neuberger, A.) 271
Glycine, quantitative utilization of, effect of cobalamin on, for synthesis of choline and methyl groups of methionine (Arnstein, H. R. V & Neuberger, A.) 259
Glycine, synthesis in rat (Arnstein, H. R. V. & Neuberger, A.) 271
Glycinebetaine in *Beta vulgaris* L. and other plants, estimation and distribution of (Cromwell, B. T. & Rennie, S. D.) 189
Glycogens, periodate oxidation of (Manners, D. J.) xx
Glycollic acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Glycolysis, in electrically stimulated cerebral-cortex slices (Heald, P. J.) 625
Glycolysis in muscle, relation to myoglobin (Lawrie, R. A.) 305
Gram-negative coccus, production of fatty acids by (Elsden, S. R. & Lewis, D.) 183
Groundnut plant, γ-amino-α-methylenebutyric acid in (Fowden, L. & Done, J.) 548

Haddock, lipids (Lovern, J. A. & Olley, J.) 686
Haemoglobin derivatives, reactions with ^{131}I-labelled *p*-iodophenylhydroxylamine and *p*-iodonitrosobenzene (Jackson, H. & Thompson, R.) xxxvi
Hair grease of dog, occurrence of two valeric acids, α- and β-methylbutyric acids in (Brouwer, E. & Nijkamp, H. J.) 444
Halogenoacetic acids, esterase hydrolysis of (Bergmann, F. & Shimoni, A.) 50
Halogenobenzenes, formation of catechols from, in rabbit (Azouz, W. M., Parke, D. V. & Williams, R. T.) 146
Heart-muscle sarcosomes, effect of calcium on respiration and phosphorylation in (Slater, E. C. & Cleland, K. W.) 566
Helix asperia, intestinal enzymes, action on fungal mycelium (Smithies, W. R.) 346
Helix pomatia, hepatopancreas of, aerobic metabolism of (Rees, K. R.) 478
Heparin, anti-thrombic action of, effect of reducing agents on (Green, J.) xxiii
Hepatopancreas of *Helix pomatia*, aerobic metabolism of (Rees, K. R.) 478
n-Hexanoate, formed from lower fatty acids by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Hexokinase in intestinal mucosa, determination of activity (Hele, M. P.) 857
Hexosamine in sheep salivary mucoid (McCrea, J. F.) 132
Hexose in fractions of bacterial cells (Fernell, W. R. & King, H. K.) 758
Hexose phosphates, concentration in rat muscle, and effects of cortisone, deoxycorticosterone and adrenalectomy (Conway, E. J. & Hingerty, D.) 447
Hexose polymers, chemical synthesis; formation during acid hydrolysis (Kent, P. W.) 361
Hippuric acid, chromatography of (Arnstein, H. R. V. & Neuberger, A.) 271
Hippuric acid formation in locust (Friedler, L. & Smith, J. N.) vi
Hippuric acids, apparent dissociation constants (Robinson, D., Smith, J. N. & Williams, R. T.) 151
Histochemistry of brain, phosphatase (Pratt, O. E.) 140
Hormones, androgenic, and ali-esterase inhibition (Myers, D. K., Simons, S. P. & Simons, E. G. L.) i
Horseradish peroxidase and cytochrome *c* peroxidase, chemical nature of second hydrogen peroxide compound formed by (George, P.) 220
Housefly *see Musca domestica*
Hyaluronate, turbidimetric estimation of (Pearce, R. H.) 472
Hyaluronidase, turbidimetric estimation of (Pearce, R. H.) 467
Hydrocarbons in fish lipids (Lovern, J. A. & Olley, J.) 686
Hydrogen, formation by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Hydrogen peroxide compound, formed by cytochrome *c* peroxidase and horseradish peroxidase (George, P.) 220
Hydrolysis, enzymic, of acid anhydrides (Bergmann, F., Wurzel, M. & Shimoni, E.) 888
Hydroperoxides, alkyl, and metmyoglobin, reaction between (George, P. & Irvine, D. H.) 230
β-Hydroxybutyric acid as a primer for fatty acid oxidation (Judah, J. D. & Rees, K. R.) 664

17-Hydroxycorticosterone, effect on uptake of sodium and potassium by yeast (Conway, E. J. & Hingerty, D.) 455
α-Hydroxy-*n*-docosanoic acid in phrenosinic acid (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 707
2-Hydroxyethane-1-sulphonic acid, fate of, in rat (Maw, G. A.) 37
β-Hydroxyethylmethyl sulphide, oxidation to sulphate in rat (Maw, G. A.) 42
α-Hydroxy-*n*-fatty acids in phrenosinic acid (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 707
α-Hydroxy-*n*-fatty acids of yeast cerebrin (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 711
α-Hydroxy-*n*-hexacosanoic acid in phrenosinic acid (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 707
α-Hydroxy-*n*-hexacosanoic acid in yeast cerebrin (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 711
11-β-Hydroxylation, enzymic, of 11-deoxycorticosterone (DOC) (Brownie, A. C., Grant, J. K. & Taylor, W.) xxi
5-Hydroxymethylcytosine in deoxyribonucleic acids (Wyatt, G. R. & Cohen, S. S.) 774
p-Hydroxyphenylacetic acid (PHPA) in urine, significance of (Aterman, K., Boscott, R. J. & Cooke, W. T.) xvii
Hydroxypyridines, glucuronic acid conjugation of (Smith, J. N.) 156
Hydroxyquinoline glucuronides, formation, adsorption spectra (Smith, J. N.) 156
3β-Hydroxysteroids, precipitation by digitonin (Haslam, R. M. & Klyne, W.) 340
α-Hydroxy-*n*-tetracosanoic acid in phrenosinic acid (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 707
α-Hydroxy-*n*-tetracosanoic acid in yeast cerebrin (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 711

Immune globulin, absorption by young lamb after ingestion of colostrum (McCarthy, E. F. & McDougall, E. I.) 177
Influenza virus receptor-substance, studies on (McCrea, J. F.) 132
Infrared spectra of bile acids (Wootton, I. D. P. & Wiggins, H. S.) 292
Inhibitor constants, enzyme, determination (Dixon, M.) 170
Inhibitors and substrates, effect of pH on affinities of enzymes for (Dixon, M.) 162
Inhibitors, effects on fumarase activity (Massey, V.) 172
Inositol in fish lipids (Lovern, J. A. & Olley, J.) 686
Insect exocuticle, hardening of, reactions probably involved (Hackman, R. H. & Todd, A. R.) 631
Insect sarcosomes, oxidative phosphorylation and effect of dinitrophenol (Lewis, S. E. & Slater, E. C.) xxvii
Insectorubin, insectoverdin, in larvae of *Plusia gamma* (Goodwin, T. W.) 834
'Intrinsic factor', Castle's, observations on chemical nature of (Latner, A. L. & McEvoy-Bowe, E.) xxiii
'Intrinsic factor' concentrates, combination of vitamin B_{12}-like compounds with (Gregory, M. E. & Holdsworth, E. S.) 830, ix
Invertase, formation in *Saccharomyces fragilis* (Davies, R.) 484
^{131}Iodine, antisera, quantitative studies with (Boursnell, J. C., Coombs, R. R. A. & Rizk, V.) 745
^{131}Iodine-labelled *p*-iodophenylhydroxylamine, reactions of haemoglobin derivatives with (Jackson, H. & Thompson, R.) xxxvi
Iodoacetate, effect on metabolism of stimulated cerebral cortex (Heald, P. J.) 625
Iodoacetate inhibition of oxidation of glycerol by myobacteria (Hunter, G. J. E.) 320
Iodomethanesulphonic acid, fate of, in rat (Maw, G. A.) 37
p-Iodonitrosobenzene, reactions of haemoglobin derivatives with (Jackson, H. & Thompson, R.) xxxvi
Iron and cobalt, effect on production of toxin by *Corynebacterium diphtheriae* (Clarke, W. A. & Clarke, G. D.) xvi
Iron in hepatic cells, effect of fasting (Harrison, M. F.) 204
Isethionic acid, fate of, in rat (Maw, G. A.) 37
Isoniazid *see iso*Nicotinic acid hydrazide

Kale, minced, fermentation mixtures of, breakdown of amino acids in (Robertson, H. A. & Barnett, A. J. G.) xx
Keto acids, production from acetate by a vibrio (Dagley, S. & Patel, M. D.) xxxvi
α-Keto acids, separation in blood and urine by paper chromatography (Smith, M. J. H. & Taylor, K. W.) xxx
α-Ketoglutarate, oxidation of, coupled with oxidative phosphorylation, by heart-muscle sarcosomes (Slater, E. C. & Holton, F. A.) 530
α-Ketoglutarate oxidized by mitochondria (Chappell, J. B. & Perry, S. V.) 586
α-Ketoglutarate, active transport by kidney-cortex slices (Whittam, R. & Davies, R. E.) 880
α-Ketoglutaric oxidase, heat inactivation of (Slater, E. C. & Cleland, K. W.) 566
α-Ketoglutaric oxidase in *Pseudomonas fluorescens* (Kogut, M. & Podoski, E. P.) 800
Kidney mitochondria, activation of fatty acid oxidation by (Judah, J. D. & Rees, K. R.) 664
Kinetics, apparatus for study of (Dalziel, K.) 79
Kinetics of oxidative phosphorylation reaction and adenine nucleotide specificity (Slater, E. C. & Holton, F. A.) 530

Lactate, metabolism by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Lactate, oxidation by stimulated cerebral cortex (Heald, P. J.) 625
Lactic acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Lactic acid, determination in microgram quantities (Hullin, R. P. & Noble, R. L.) 289
Lactobacillus arabinosus, nicotinamide deamidase in (Hughes, D. E. & Williamson, D. H.) 851
Lactobacillus casei, hydrolysates of, optical form of glutamic acid in (Wiltshire, G. H.) 46
β-Lactoglobulin isoelectric, osmotic pressures of (Smithies, O.) 57
Lactose, synthesis from glucose in mammary gland (Reiss, O. K. & Barry, J. M.) 783
Lamb, absorption of immune globulin by (McCarthy, E. F. & McDougall, E. I.) 177
Lard, vitamin A-replacing effect of (Lowe, J. S. & Morton, R. A.) 681
Lead dithionate, removal of excess periodate by (O'Dea, J. F. & Gibbons, R. A.) 580
Lecithin in fish lipids (Lovern, J. A. & Olley, J.) 686
Lecithinase C, study of action of, evidence for structure of ovolecithin derived from (Long, C. & Maguire, M. F.) xv
Limpet, preparation of arylsulphatase and β-glucuronidase concentrates from (Dodgson, K. S. & Spencer, B.) 315
Lipase, oat (Martin, H. F. & Peers, F. G.) 523
Lipid in hepatic cells, effect of fasting (Harrison, M. F.) 204
Lipid material from human placenta, fractionation of, application of countercurrent methods to (Cole, P. G., Lathe, G. H. & Ruthven, C. R. J.) 17
Lipids from urines of bulls and pregnant cows (Broadbent, I. E., Brooks, R. V. & Klyne, W.) vi
Lipids of brain dispersion, incorporation of labelled phosphate into (Dawson, R. M. C.) 507
Lipids of fish (Lovern, J. A. & Olley, J.) 686
Lipoprotein complex, particulate, association of alkaline phosphatase of milk with (Morton, R. K.) 786
Littorina littorea, sulphatases and glucuronidases of (Dodgson, K. S., Lewis, J. I. M. & Spencer, B.) 253
Liver cell, effect of starvation on composition of (Harrison, M. F.) 204
Liver glutathione level, changes in dietetic liver necrosis (Lindan, O. & Work, E.) 554

Liver mitochondria, activation of fatty acid oxidation by (Judah, J. D. & Rees, K. R.) 664
Liver necrosis, experimental, in rats (Lindan, O. & Work, E.) 554, 562
Liver, rat, properties and assay of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of (Glock, G. E. & McLean, P.) 400
Locust, hippuric acid formation in (Friedler, L. & Smith, J. N.) vi
Lutein in larvae of *Plusia gamma* (Goodwin, T. W.) 834
Lycium chinense Mill. leaves, glycinebetaine content of (Cromwell, B. T. & Rennie, S. D.) 189
Lysine polymers, action of trypsin on (Waley, S. G. & Watson, J.) 328

Malate oxidized by mitochondria (Chappell, J. B. & Perry, S. V.) 586
Maleic acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Malic acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Malic dehydrogenase in *Pseudomonas fluorescens* (Kogut, M. & Podcski, E. P.) 800
Malonate, effect on metabolism of stimulated cerebral cortex (Heald, P. J.) 625
Malonic acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Maltose, action of mould enzymes on (Bealing, F. J.) 93
Mammary gland homogenates, synthesis and oxidation of citric acid in (Terner, C.) xxix
Mammary gland, synthesis of lactose from glucose in (Reiss, O. K. & Barry, J. M.) 783
Mammary tissue *in vitro*, lactating sheep, oxidation of glucose and acetate by (Duncombe, W. G. & Glascock, R. F.) xxiii
Man, bile acids of (Wootton, I. D. P. & Wiggins, H. S.) 292
Manganese and plant peroxidases (Kenten, R. H.) 350
Mannitol, metabolism by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Melanin in larvae of *Plusia gamma* (Goodwin, T. W.) 834
Melezitose, action of mould enzymes on (Bealing, F. J.) 93
Mercapturic acids, apparent dissociation constants (Robinson, D., Smith, J. N. & Williams, R. T.) 151
Mesobiliverdin in larvae of *Plusia gamma* (Goodwin, T. W.) 834
Metabolism, carbohydrate, in *Escherichia coli* (McNair Scott, D. B. & Cohen, S. S.) 23, 33
Metabolism, in cerebral tissues (McIlwain, H.) 618
Metals of group II of periodic classification, stabilities of complexes formed with, effect of amino acid structure on (Perkins, D. J.) 649
Methaemoglobin, spectrophotometric study of ionizations in (George, P. & Hanania, G.) 236
Methanesulphonic acid, fate of, in rat (Maw, G. A.) 37
DL-Methionine and biosynthesis of choline by *Cicer arietinum* (Ahmad, K. & Karim, M. A.) 817
Methionine, choline and methyl groups of, synthesis of, effect of cobalamin on quantitative utilization of serine, glycine and formate for (Arnstein, H. R. V. & Neuberger, A.) 259
Methionine, synthesized by *Bacterium coli*, effect of growth on medium containing (Wijesundera, S. & Woods, D. D.) viii
Methyl groups of methionine, synthesis of (Arnstein, H. R. V. & Neuberger, A.) 259
Methylbutyric acids, α- and β-, occurrence in hair grease of dog (Brouwer, E. & Nijkamp, H. J.) 444
α-Methylglucoside, action of mould enzymes on (Bealing, F. J.) 93
Metmyoglobin and alkyl hydroperoxides, reaction between (George, P. & Irvine, D. H.) 230
Micro-organisms, biochemistry of (Raistrick, H., Stickings, C. E. & Thomas, R.) 421
Microsporum, effect of inhibitors on metabolism of (Barlow, A. J. E., Chattaway, F. W. & Thompson, C. C.) xxxi
Milk, alkaline phosphatase of (Morton, R. K.) 786, 795
Milk fats of *Balaenoptera physalus* (finner whale), component acids of (Cama, J. S. & Meara, M. L.) 365
Milk whey, sow's, combination of vitamin B_{12}-like compounds with (Gregory, M. E. & Holdsworth, E. S.) 830
Mitochondria, kidney and liver, activation of fatty acid oxidation by (Judah, J. D. & Rees, K. R.) 664
Mitochondria, skeletal-muscle, respiration and adenosinetriphosphatase activities of (Chappell, J. B. & Perry, S. V.) 586
Molecular weight of purified sheep salivary mucoid (Caspary, E. A.) 139
Molluscs, marine, arylsulphatase and β-glucuronidase of (Dodgson, K. S., Lewis, J. I. M. & Spencer, B.) 253
Molybdate, catalytic effect of, on hydrolysis of organic phosphates (Weil-Malherbe, H.) 741
Monofluoroacetate on metabolism of *Rhodospirillum rubrum* (Elsden, S. R. & Ormerod, J. G.) iii
Mould 'glucosaccharase': a fructosidase (Bealing, F. J.) 93
Mucoid from salivary gland of sheep, physicochemical examination (Caspary, E. A.) 139
Mucoid, homogeneous, from salivary gland of sheep, preparation and properties (McCrea, J. F.) 132
Mucosa, intestinal, hexokinase activity in (Hele, M. P.) 857
Musca domestica, phosphorus metabolism in (Wintering-ham, F. P. W., Loveday, P. M. & Hellyer, G. C.) xxxiii
Muscle, activity of cytochrome system in (Lawrie, R. A.) 298
Muscle, fish skeletal, proteins of (Connell, J. J.) 378
Muscle mitochondria, skeletal, respiration and adenosinetriphosphatase activities of (Chappell, J. B. & Perry, S. V.) 586
Muscle, phosphate esters of, separation by paper chromatography (Caldwell, P. C.) 458
Muscle, proteins of (Perry, S. V.) 114
Muscle, relation of energy-rich phosphate in, to myoglobin and cytochrome-oxidase activity (Lawrie, R. A.) 305
Muscle sarcosomes, heart, effect of calcium on respiration and phosphorylation in (Slater, E. C. & Cleland, K. W.) 566
Muscle sarcosomes, heart, oxidative phosphorylation coupled with oxidation of α-ketoglutarate by (Slater, E. C. & Holton, F. A.) 530
Mycelium of *Penicillium griseofulvum* Dierckx, lysing action of enzymes on (Smithies, E. R.) 346
Mycobacteria, oxidation of glycerol by (Hunter, G. J. E.) 320
Mycobacterium johnei, mycobactin a growth factor for (Francis, J., Macturk, H. M., Madinaveitia, J. & Snow, G. A.) 596
Mycobacterium phlei, mycobactin isolation from (Francis, J., Macturk, H. M., Madinaveitia, J. & Snow, G. A.) 596
Mycobactin, isolation and properties (Francis, J., Macturk, H. M., Madinaveitia, J. & Snow, G. A.) 596
Myofibril, isolated, proteins of (Perry, S. V.) 114
Myoglobin, activity of cytochrome system in muscle and relation to (Lawrie, R. A.) 298
Myoglobin, relation of energy-rich phosphate in muscle to (Lawrie, R. A.) 305
Myokinase and oxidative phosphorylation in heart-muscle sarcosomes (Slater, E. C. & Holton, F. A.) 530

Necrosis, experimental liver, in rats (Lindan, O. & Work, E.) 554, 562
Nicotinamide deamidase, occurrence in bacteria (Hughes, D. E. & Williamson, D. H.) 851
Nicotinamide, deamidation by bacteria (Hughes, D. E. & Williamson, D. H.) 851
*iso*Nicotinic acid hydrazide, metabolites of, detection and identification in human urine (Cuthbertson, W. F. J., Ireland, D. M. & Wolff, W.) 669

Nitrate and biosynthesis of choline by *Cicer arietinum* (Ahmad, K. & Karim, M. A.) 817
Nitrogen in fractions of bacterial cells (Fernell, W. R. & King, H. K.) 758
Nitrogen metabolism in *Pisum sativum* (Hyde, T. G.) xxi
Nitrophenols, metabolism by bacteria (Simpson, J. R. & Evans, W. C.) xxiv
Nodules from rheumatic fever patients (Consden, R., Glynne, L. E. & Stanier, W. M.) 248
Nucleic acid in fractions of bacterial cells (Fernell, W. R. & King, H. K.) 758
Nucleic acids, incorporation of radioactive phosphate into, in brain slices (Deluca, H. A., Rossiter, R. J. & Strickland, K. P.) 193
5-Nucleotidase in brain (Pratt, O. E.) 140
Nucleotide of myofibril (Perry, S. V.) 114

Oat lipase (Martin, H. F. & Peers, F. G.) 523
Octanoic acid, oxidation in liver and kidney mitochondria (Judah, J. D. & Rees, K. R.) 664
Oligosaccharides, formation during enzymic hydrolysis of β-glucosides (Crook, E. M. & Stone, B. A.) xxv
Oligosaccharides from *Agave vera cruz* Mill. (Srinivasan, M. & Bhatia, I. S.) 286
Osmometer, dynamic, accurate measurements for small volumes (Smithies, O.) 57
Ovolecithin, evidence for structure of, derived from study of action of lecithinase C (Long, C. & Maguire, M. F.) xv
Ox liver, sulphatase of (Roy, A. B.) 653
Ox *see also* Bulls and Cows
Oxaloacetic decarboxylase in *Pseudomonas fluorescens* (Kogut, M. & Podoski, E. P.) 800
Oxidase, polyphenol, from potato, *in vitro* activity, effect of L-ascorbic acid on (Baruah, P. & Swain, T.) 392
Oxidation, fatty acid, activation by kidney and liver mitochondria (Judah, J. D. & Rees, K. R.) 664
Oxidative phosphorylation and incorporation of phosphate into brain lipids (Dawson, R. M. C.) 507
Oxidative phosphorylation coupled with oxidation of α-ketoglutarate by heart-muscle sarcosomes (Slater, E. C. & Holton, F. A.) 530
Oxidative phosphorylation, kinetics of (Slater, E. C. & Holton, F. A.) 530
α-Oxo acids, monobasic, isomerism of 2:4-dinitrophenylhydrazones of (Stewart, H. B.) xxvi
Oxo acids *see also* Keto acids
α-Oxoglutarate *see* α-Ketoglutarate
Oxygen consumption, effect of polyamino acids on (Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E.) 671

Pantothenate, effect on ester cholesterol of rats (Boyd, G. S.) 892
Paper chromatography, separation of α-keto acids by, in blood and urine (Smith, M. J. H. & Taylor, K. W.) xxx
Paper chromatography, separation of uroporphyrin esters I and III by (Falk, J. E. & Benson, A.) 101
Paper, continuous electrophoresis on (Holdsworth, E. S.) xiv
Patella vulgata, sulphatases and glucuronidases of (Dodgson, K. S., Lewis, J. I. M. & Spencer, B.) 253
Patella vulgata, *see also* Limpet
Pea-seedling tissues and extracts, oxidation of tryptophan in, association with peroxidase (Wiltshire, G. H.) 408
Penicillin, biosynthesis (Arnstein, H. R. V. & Grant, P.) v
Penicillium griseofulvum Dierckx, mycelium of, lysing action of enzymes on (Smithies, W. R.) 346
Penicillium spinulosum, action on oligosaccharides (Bealing, F. J.) 93
n-Pentanoate, formed from lower fatty acids by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Pentose in fractions of bacterial cells (Fernell, W. R. & King, H. K.) 758
Pentosenucleic acids of brain slices, factors affecting incorporation of radioactive phosphate into (Findlay, M., Rossiter, R. J. & Strickland, K. P.) 200
Peptides, action of trypsin on (Waley, S. G. & Watson, J.) 328
Peptides, simple, complexes with zinc and cadmium ions in aqueous solution (Perkins, D. J.) xxviii
Periodate oxidation of carbohydrates, estimation of formaldehyde liberated during (O'Dea, J. F. & Gibbons, R. A.) 580
Periodate oxidation of glycogens (Manners, D. J.) xx
Peroxidase and oxidation of tryptophan in pea-seedling tissues and extracts (Wiltshire, G. H.) 408
Peroxidase, cytochrome *c*, and horseradish peroxidase, chemical nature of second hydrogen peroxide compound formed by (George, P.) 220
Peroxidase, horseradish, oxidation of phenylacetaldehyde by (Kenten, R. H.) 350
Peroxide compound, second hydrogen, chemical nature of, formed by cytochrome *c* peroxidase and horseradish peroxidase (George, P.) 220
[^{14}C]Phenol, metabolism of (Parke, D. V. & Williams, R. T.) 337
Phenolic acids in rat, effect of dietary deficiencies, aminopterin, ascorbic acid, chloretone and antibiotics on (Boscott, R. J. & Greenberg, S. M.) xviii
Phenylacetaldehyde, oxidation of, by plant saps (Kenten, R. H.) 350
Phenylalanine, inhibition of incorporation in *Staphylococcus aureus* by chloramphenicol and *p*-chlorophenylalanine (Gale, E. F. & Folkes, J. P.) 730
Phenylglucuronide, formation from [^{14}C]benzene (Parke, D. V. & Williams, R. T.) 337
Phenylsulphuric acid, formation from [^{14}C]benzene (Parke, D. V. & Williams, R. T.) 337
Phloroglucinol reaction for galactose, non specific (Fowweather, F. S.) 718
Phosphatase activity in brain, histochemistry of (Pratt, O. E.) 140
Phosphatase activity, proportion demonstrable in brain by histological techniques (Pratt, O. E.) 140
Phosphatase, alkaline, of milk (Morton, R. K.) 786, 795
Phosphatase, effect of pH on Michaelis constants (Dixon, M.) 162
Phosphatases of a brain (Gordon, J. J.) 812
Phosphate, energy-rich, relation in muscle to myoglobin and cytochrome-oxidase activity (Lawrie, R. A.) 305
Phosphate esters of muscle, separation by paper chromatography (Caldwell, P. C.) 458
Phosphate, radioactive, incorporation into lipids of brain dispersion (Dawson, R. M. C.) 507
Phosphate, radioactive, incorporation into nucleic acids of brain slices (Deluca, H. A., Rossiter, R. J. & Strickland, K. P.) 193
Phosphate, radioactive, incorporation into pentosenucleic acids of brain slices (Findlay, M., Rossiter, R. J. & Strickland, K. P.) 200
Phosphates, aromatic *see* Tri-*o*-cresyl
Phosphates, organic, determination of (Weil-Malherbe, H.) 741
Phosphates, organic, hydrolysis, catalytic effect of molybdate (Weil-Malherbe, H.) 471
Phosphates, sugar, dehydrogenases of (Glock, G. E. & McLean, P.) 400
Phosphatidylethanolamine in fish lipids (Lovern, J. A. & Olley, J.) 686
Phosphocreatine, concentration in rat muscle, and effects of cortisone, deoxycorticosterone and adrenalectomy (Conway, E. J. & Hingerty, D.) 447
6-Phosphogluconate dehydrogenase, isolation and properties (McNair Scott, D. B. & Cohen, S. S.) 23
6-Phosphogluconate dehydrogenase of rat liver, properties and assay (Glock, G. E. & McLean, P.) 400

6-Phosphogluconate dehydrogenase, quantitative studies (McNair Scott, D. B. & Cohen, S. S.) 33
Phospholipid metabolism, influence of energy intake on (Wikramanayake, T. W., Munro, H. N., Naismith, D. J. & Hutchison, W. C.) 640
Phospholipids, cerebral, labelled with ^{32}P, complete fractionation of (Dawson, R. M. C.) xii
Phospholipids of placenta (Cole, P. G., Lathe, G. H. & Ruthven, C. R. J.) 17
Phosphorus compounds, organic, mechanism of inhibition of cholinesterases by (Aldridge, W. N. & Davison, A. N.) 763
Phosphorus in fractions of bacterial cells (Fernell, W. R. & King, H. K.) 758
Phosphorus labelled adenosine triphosphate (Hems, R. & Bartley, W.) 434
Phosphorus metabolism in *Musca domestica* (Wintering-ham, F. P. W., Loveday, P. M. & Hellyer, G. C.) xxxiii
Phosphorylation and anaerobic breakdown of citrulline (Knivett, V. A.) x
Phosphorylation, enzymic, of D-ribose (Heald, K. & Long, C.) xv
Phosphorylation in heart-muscle sarcosomes, effect of calcium (Slater, E. C. & Cleland, K. W.) 566
Phosphorylation in oxidation of glycerol by mycobacteria (Hunter, G. J. E.) 320
Phosphorylation of sugars in intestinal mucosa, glucose, fructose, mannose, xylose and galactose (Hele, M. P.) 857
Phosphorylation of sugars in rat (Hele, M. P.) 857, 864
Phosphorylation, oxidative, coupled with oxidation of α-ketoglutarate by heart-muscle sarcosomes (Slater, E. C. & Holton, F. A.) 530
Phosphorylation, oxidative, in insect sarcosomes (Lewis, S. E. & Slater, E. C.) xxvii
Photoelectric spectrophotometry, precision of analysis by (Dalziel, K.) 90
Photometer, direct-reading flame, precision of, for determination of sodium and potassium in biological fluids (Holiday, E. R. & Preedy, J. R. K.) 214
Phrenosinic acid, further observations on (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 707
Pigments, diazo reacting, of serum, urine and bile (Cole, P. G., Lathe, G. H. & Billing, B. H.) xiii
Pisum sativum, nitrogen metabolism in (Hyde, T. G.) xxi
Placenta, human, fractionation of lipid material from, application of countercurrent methods to (Cole, P. G., Lathe, G. H. & Ruthven, C. R. J.) 17
Plant saps, oxidation of phenylacetaldehyde by (Kenten, R. H.) 350
Plants, biosynthesis and metabolism of betaines in (Cromwell, B. T. & Rennie, S. D.) 189
Plasma, free and combined cholesterol in, chromatography of (Kerr, L. M. H. & Bauld, W. S.) 872
Plasma, human, fibrinolysins of (Bidwell, E.) 497
Plasmalogens in fish lipids (Lovern, J. A. & Olley, J.) 686
Plasmin prepared by activation with chloroform, comparison with fibrinolytic plasma from normal subjects and cadaver blood (Bidwell, E.) 497
Plusia gamma, pigments in larvae of (Goodwin, T. W.) 834
Poly-α-amino acids, water-soluble, action on bacteria (Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E.) 671
Poly-DL-arginine, inhibition of bacteria by (Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E.) 671
Polyfructosans from *Agave vera cruz* Mill. (Srinivasan, M. & Bhatia, I. S.) 286
Polyglucose A, sedimentation coefficient of (Baldwin, R. L.) 644
Polylysine, action of trypsin on (Waley, S. G. & Watson, J.) 328
Poly-L-lysine, inhibition of bacteria by (Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E.) 671
Polymyxin B, antibacterial activity, reversal by non-toxic divalent cations (Newton, B. A.) x
Poly-DL-ornithine, inhibition of bacteria by (Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E.) 671
Polyphenoloxidase from potato, *in vitro* activity, effect of L-ascorbic acid on (Baruah, P. & Swain, T.) 392
Polysaccharide in nodules from rheumatic fever patients (Consden, R., Glynn, L. E. & Stanier, W. M.) 248
Polysaccharide, stabilization of elastin by (Wood, G. C.) xxxiv
Porphyria, cutaneous, acute and congenital, uroporphyrins from (Canivet, J. & Rimington, C.) 867
Porphyria urines, acute, 'Waldenström' porphyrin of (Kennard, O. & Rimington, C.) 105; (Nicholas, R. E. H. & Rimington, C.) 109
Porphyrin synthesis by *Rhodopseudomonas spheroides* (Lascelles, J.) iv
Porphyrins, identification and classification, on basis of their X-ray diffraction patterns (Kennard, O. & Rimington, C.) 105
Potassium, active transport by kidney-cortex slices (Whittam, R. & Davies, R. E.) 880
Potassium and biosynthesis of choline by *Cicer arietinum* (Ahmad, K. & Karim, M. A.) 817
Potassium and sodium in biological fluids, precision of direct-reading flame photometer for determination of (Holiday, E. R. & Preedy, J. R. K.) 214
Potassium as source of inaccuracy in chemical estimation of sodium in urine (Aitken, E. H. & Preedy, J. R. K.) 211
Potassium, concentration in rat muscle, and effects of cortisone, deoxycorticosterone and adrenalectomy (Conway, E. J. & Hingerty, D.) 447
Potassium in hepatic cells, effect of fasting (Harrison, M. F.) 204
Potassium ions in yeast, active transport of, effects of cortisone, deoxycorticosterone and other steroids on (Conway, E. J. & Hingerty, D.) 455
Potato, *in vitro* activity of polyphenoloxidase from, effect of L-ascorbic acid on (Baruah, P. & Swain, T.) 392
Pregnanediol glucuronide, oxidative fission of, with sodium bismuthate (Norymberski, J. K. & Sermin, A. T.) 876
Procollagen, reaction with fluorodinitrobenzene (Bowes, J. H. & Moss, J. A.) 735
Protein in hepatic cells, effect of fasting (Harrison, M. F.) 204
Protein solutions, osmometer for study of (Smithies, O.) 57
Protein synthesis in bacteria (Gale, E. F. & Folkes, J. P.) 721, 730
Protein synthesis in rat brain (Gaitonde, M. K. & Richter, D.) viii
Protein synthesis, inhibition by antibiotics (Gale, E. F. & Folkes, J. P.) 721
Proteins, cerebrospinal fluid, separation by paper electrophoresis (Eaton, J. C. & Gardner, M. D.) xxv
Proteins, estimation of D- and L-glutamic acid in (Wiltshire, G. H.) 46
Proteins, hydrolysates of, optical form of glutamic acid in (Wiltshire, G. H.) 46
Proteins of barley, cytoplasmic and chloroplastic, amino acids of (Yemm, E. W. & Folkes, B. F.) 700
Proteins of fish skeletal muscle (Connell, J. J.) 378
Proteins of isolated myofibril (Perry, S. V.) 114
Proteins, reaction with fluorodinitrobenzene (Bowes, J. H. & Moss, J. A.) 735
Proteins, soluble, role in elimination of carcinogens from animal body (Chalmers, J. G.) xix
Proteinuria of lambs given colostrum (McCarthy, E. F. & McDougall, E. I.) 177
Protoporphyrin, paper chromatography of (Falk, J. E. & Benson, A.) 101
Pseudocholinesterases, species variation in specificity pattern of (Myers, D. K.) 67

Pseudomonas fluorescens, oxidative pathways in (Kogut, M. & Podoski, E. P.) 800
Pyridine derivatives, identification and estimation of (Hughes, D. E. & Williamson, D. H.) 851
Pyrimidine and citrulline, required for growth of a strain of *Bacterium coli* (Back, K. J. C. & Woods, D. D.) xii
Pyrophosphatase in brain (Gordon, J. J.) 812
Pyrophosphate in muscle extracts (Caldwell, P. C.) 458
Pyruvate, as metabolite of cystine (Arnstein, H. R. V. & Crawhall, J. C.) 280
Pyruvate in oxidation of glycerol by mycobacteria (Hunter, G. J. E.) 320
Pyruvate, metabolism by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Pyruvate, oxidation by breis and slices of pigeon brain, accumulation of citrate during (Coxon, R. V.) 545
Pyruvic acid, removal of interference in ultramicrodetermination of lactic acid (Hullin, R. P. & Noble, R. L.) 289

Rabbits, immune and normal, fate of intravenously injected ^{131}I-labelled antigens and 3:5-diiodotyrosine in (Almond, A. E., Francis, G. E., Hawkins, J. D. & Wormall, A.) xxxiii
Raffinase, formation in *Saccharomyces fragilis* (Davies, R.) 484
Raffinose, action of mould enzymes on (Bealing, F. J.) 93
Raffinose, fermentation by *Saccharomyces fragilis* (Davies, R.) 484
Rapid reactions, spectrophotometric study (Dalziel, K.) 79
Rat, cholesterol in, possible role of coenzyme A in biosynthesis of (Boyd, G. S.) 892
Rat, excretion in urines of phenolic acids in, effect of dietary deficiencies, aminopterin, ascorbic acid, chloretone and antibiotics on (Boscott, R. J. & Greenberg, S. M.) xviii
Rat, fate of some aliphatic sulphonic acids in (Maw, G. A.) 37
Rat, oxidation of dimethylthetin and related compounds in (Maw, G. A.) 42
Rat, sexual organs of, 7-dehydrosterol in (Ward, R. J. & Moore, T.) 295
Rat, sugars in, phosphorylation and absorption of (Hele, M. P.) 857, 864
Rat, synthesis of glycine and serine in (Arnstein, H. R. V. & Neuberger, A.) 271
Rats, carotene injected into, site of conversion of (Bieri, J. G. & Pollard, C. J.) xxxi
Rats, influence of cobalamin on growth on diets deficient in labile methyl groups (Arnstein, H. R. V. & Neuberger, A.) 259
Receptor-substance, influenza virus, studies on (McCrea, J. F.) 132
Red-cell antigens (Boursnell, J. C., Coombs, R. R. A. & Rizk, V.) 745
Refractive-index gradient curve, methods of measurement of sedimentation coefficients based on (Baldwin, R. L.) 644
Refractometric data and calculation of mole fractions (Smithies, O.) 57
Resorcinol-4:6-disulphonic acid, as colorimetric reagent for carbohydrates (Lunt, E. & Sutcliffe, D.) 122
Respiration, effect of metabolic inhibitors on, in electrically stimulated cerebral-cortex slices (Heald, P. J.) 625
Respiration in heart-muscle sarcosomes, effect of calcium (Slater, E. C. & Cleland, K. W.) 566
Respiration of skeletal-muscle mitochondria (Chappell, J. B. & Perry, S. V.) 586
Rheumatic fever, chemical examination of connective tissue in (Consden, R., Glynn, L. E. & Stanier, W. M.) 248
Rhodopseudomonas spheroides, porphyrin synthesis by (Lascelles, J.) iv
Rhodospirillum rubrum, monofluoroacetate on metabolism of (Elsden, S. R. & Ormerod, J. G.) iii
Riboflavin and biosynthesis of choline by *Cicer arietinum* (Ahmad, K. & Karim, M. A.) 817
D-Ribose, enzymic phosphorylation of (Heald, K. & Long, C.) xv
Rotations, molecular, of triterpenoid alcohols and their derivatives (Klyne, W. & Stokes, W. M.) xxviii
Rumen, β-glucosidase activity, inhibition by glucono-1:5-lactone (Conchie, J.) xxi

Saccharase, effect of pH on Michaelis constants (Dixon, M.) 162
Saccharomyces fragilis, enzyme formation in (Davies, R.) 484
Salivary gland of sheep, mucoid from, physicochemical examination (Caspary, E. A.) 139
Salivary gland of sheep, preparation and properties of homogeneous mucoid from (McCrea, J. F.) 132
Salmine, amino acid composition of (Corfield, M. C. & Robson, A.) 517
Sarcosomes, heart-muscle, effect of calcium on respiration and phosphorylation in (Slater, E. C. & Cleland, K. W.) 566
Sarcosomes, heart-muscle, oxidative phosphorylation coupled with oxidation of α-ketoglutarate by (Slater, E. C. & Holton, F. A.) 530
Sarcosomes, insect, effect of dinitrophenol on (Slater, E. C. & Lewis, S. E.) xxvii
Sarcosomes, insect, oxidative phosphorylation in (Lewis, S. E. & Slater, E. C.) xxvii
Sebum, squalene in (Wheatley, V. R.) 637
Sedimentation coefficient of polyglucose A (Baldwin, R. L.) 644
Sedimentation coefficients of small molecules: methods of measurement based on refractive-index gradient curve (Baldwin, R. L.) 644
Semen, human, zinc and carbonic anhydrase in (Mawson, C. A. & Fischer, M. I.) 696
Serine, *N*-benzoyl derivative, chromatography of (Arnstein, H. R. V. & Neuberger, A.) 271
Serine, degradation for assay of radioactivity of C atoms (Arnstein, H. R. V. & Neuberger, A.) 271
DL-Serine, metabolism by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Serine of placenta (Cole, P. G., Lathe, G. H. & Ruthven, C. R. J.) 17
Serine, quantitative utilization of, effect of cobalamin on, for synthesis of choline and methyl groups of methionine (Arnstein, H. R. V. & Neuberger, A.) 259
Serine, synthesis in rat (Arnstein, H. R. V. & Neuberger, A.) 271
Serum, determination of sodium and potassium in (Holiday, E. R. & Preedy, J. R. K.) 214
Serum, diazo reacting pigment of (Cole, P. G., Lathe, G. H. & Billing, B. H.) xiii
Serum γ-globulin, iodine uptake by (Boursnell, J. C., Coombs, R. R. A. & Rizk, V.) 745
Serum globulin of lambs given colostrum (McCarthy, E. F. & McDougall, E. I.) 177
Serum proteins of lamb given colostrum (McCarthy, E. F. & McDougall, E. I.) 177
Sexual organs of rat, 7-dehydrosterol in (Ward, R. J. & Moore, T.) 295
Sheep mammary tissue *in vitro*, lactating, oxidation of glucose and acetate by (Duncombe, W. G. & Glascock, R. F.) xxiii
Sheep salivary gland, mucoid from, physicochemical examination (Caspary, E. A.) 139
Sheep salivary gland, preparation and properties of homogeneous mucoid from (McCrea, J. F.) 132
Silage, field, occurrence of volatile fatty acids in (Barnett, A. J. G. & Duncan, R. E. B.) xix

Silver-Y moth *see Plusia gamma*
Simultaneous adaptation experiments, interpretation of (Kogut, M. & Podoski, E. P.) 800
Skeletal-muscle mitochondria, respiration and adenosine-triphosphatase activities of (Chappell, J. B. & Perry, S. V.) 586
Snail, intestinal enzymes, action on fungal mycelium (Smithies, W. R.) 346
Sodium, active transport by kidney-cortex slices (Whittam, R. & Davies, R. E.) 880
Sodium and potassium in biological fluids, precision of direct-reading flame photometer for determination of (Holiday, E. R. & Preedy, J. R. K.) 214
Sodium, concentration in rat muscle, and effects of cortisone, deoxycorticosterone and adrenalectomy (Conway, E. J. & Hingerty, D.) 447
Sodium fluoride, effect on metabolism of stimulated cerebral cortex (Heald, P. J.) 625
Sodium in urine, chemical estimation of, potassium as source of inaccuracy in (Aitken, E. H. & Preedy, J. R. K.) 211
Sodium ions in yeast, active transport of, effects of cortisone, deoxycorticosterone and other steroids on (Conway, E. J. & Hingerty, D.) 455
Sodium malonate, effect on metabolism of stimulated cerebral cortex (Heald, P. J.) 625
Sorbitol, metabolism by Gram-negative coccus (Elsden, S. R. & Lewis, D.) 183
Sow's whey, combination with vitamin B_{12}-like compounds (Gregory, M. E. & Holdsworth, E. S.) ix
Specificity, species and organ, of cholinesterases (Myers, D. K.) 67
Spectra, infrared, of bile acids (Wootton, I. D. P. & Wiggins, H. S.) 292
Spectrokinetic study of rapid reactions, apparatus for (Dalziel, K.) 79
Spectrophotometric study of ionizations in methaemoglobin (George, P. & Hanania, G.) 236
Spectrophotometry, photoelectric, precision of analysis (Dalziel, K.) 90
Spectroscopic studies of carbohydrates (Love, R. M.) 126
Spermatozoa, zinc in (Mawson, C. A. & Fischer, M. I.) 696
Spinacea oleracea L. leaves, glycinebetaine content of (Cromwell, B. T. & Rennie, S. D.) 189
Spleen glutathione level, changes in dietetic liver necrosis (Lindan, O. & Work, E.) 554
Squalene, chromatography on alumina (Wheatley, V. R.) 637
Squalene in sebum (Wheatley, V. R.) 637
Squalene, Liebermann-Burchard reaction (Wheatley, V. R.) 637
Stachyose, action of mould enzymes on (Bealing, F. J.) 93
Stachyose, preparation from *Stachys sylvatica* (Bealing, F. J.) 93
Stachys sylvatica, stachyose prepared from (Bealing, F. J.) 93
Staphylococci, fragmented, amino acid incorporation by (Gale, E. F. & Folkes, J. P.) xi
Staphylococcus albus, nicotinamide deamidase in (Hughes, D. E. & Williamson, D. H.) 851
Staphylococcus aureus, amino acid metabolism of (Gale, E. F. & Folkes, J. P.) 721, 730
Staphylococcus aureus, inhibition of, by polyamino acids (Katchalski, E., Bichowski-Slomnitzki, L. & Volcani, B. E.) 671
Staphylococcus aureus, protein synthesis in (Gale, E. F. & Folkes, J. P.) 721, 730
Starvation, effect on composition of liver cell (Harrison, M. F.) 204
Steroids, adrenal, effect on organic and inorganic metabolism (Conway, E. J. & Hingerty, D.) 447
Steroids, cortical, oxidation with sodium bismuthate (Brooks, C. J. W. & Norymberski, J. K.) 371
Steroids, effect on active transport of sodium and potassium ions in yeast (Conway, E. J. & Hingerty, D.) 455
Steroids, precipitation by digitonin (Haslam, R. M. & Klyne, W.) 340
Steroids, separation of 5-epimers (Haslam, R. M. & Klyne, W.) 340
Sterol, 7-dehydro, in sexual organs of rat (Ward, R. J. & Moore, T.) 295
Substrates and inhibitors, effect of pH on affinities of enzymes for (Dixon, M.) 162
Succinate oxidation by mitochondria (Chappell, J. B. & Perry, S. V.) 586
Succinate, oxidation by *Pseudomonas fluorescens* (Kogut, M. & Podoski, E. P.) 800
Succinic acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Succinic dehydrogenase in muscle; activity in relation to myoglobin content, activity in relation to age of animals (Lawrie, R. A.) 298
Succinic dehydrogenase in *Pseudomonas fluorescens* (Kogut, M. & Podoski, E. P.) 800
Succinic dehydrogenase-cytochrome in muscle, relation to myoglobin (Lawrie, R. A.) 305
Succinic oxidase, effect of adenosine diphosphate and ethylenediaminetetraacetic acid (Slater, E. C. & Cleland, K. W.) 566
Succinic oxidase in muscle; activity in relation to myoglobin content, activity in relation to age of animals (Lawrie, R. A.) 298
Sucrose, action of mould enzymes on (Bealing, F. J.) 93
Sucrose, fermentation by *Saccharomyces fragilis* (Davies, R.) 484
Sucrose from *Agave vera cruz* Mill. (Srinivasan, M. & Bhatia, I. S.) 286
Sugars in rat, phosphorylation and absorption of (Hele, M. P.) 857, 864
Sugars, sulphuric acid on (Love, R. M.) 126
Sulphatase of limpets, preparation free from β-glucuronidase (Dodgson, K. S. & Spencer, B.) 315
Sulphatase of ox liver (Roy, A. B.) 653
Sulphatase, purification and properties (Roy, A. B.) 653
Sulphatases, studies on (Dodgson, K. S., Lewis, J. I. M. & Spencer, B.) 253; (Dodgson, K. S. & Spencer, B.) 315, 436
Sulphate, inorganic, determination of, in study of sulphatases (Dodgson, K. S. & Spencer, B.) 436
Sulphate, oxidation of dimethylthetin and related compounds in rat to (Maw, G. A.) 42
Sulphocholine chloride, oxidation to sulphate in rat (Maw, G. A.) 42
Sulphonic acids, aliphatic, fate of, in rat (Maw, G. A.) 37
Sulphuric acid on sugars (Love, R. M.) 126
Sulphydryl groups, activation of glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase by (Glock, G. E. & McLean, P.) 400

'Taka diastase', action oligosaccharides (Bealing, F. J.) 93
Tartaric acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Techniques in tissue metabolism (Ayres, P. J. W. & McIlwain, H.) 607
Thetins, oxidation to sulphate in rat (Maw, G. A.) 42
Thiamine and biosynthesis of choline by *Cicer arietinum* (Ahmad, K. & Karim, M. A.) 817
Thioglycollic acid and derivatives, oxidation to sulphate in rat (Maw, G. A.) 42
Threonine, α-benzamido acids, chromatography of; *N*-benzoyl derivative, chromatography of (Arnstein, H. R. V. & Neuberger, A.) 271
Tissue metabolism, techniques in (Ayres, P. J. W. & McIlwain, H.) 607

Tobacco-mosaic-virus-protein, hydrolysates of, optical form of glutamic acid in (Wiltshire, G. H.) 46
Tocopherol deficiency produced by tri-*o*-cresyl phosphate (Myers, D. K., Mulder, H. E. W. & Simons, E. G. L.) i
Tollens reaction *see* Phloroglucinol reaction
o-Tolylhydrazine, in detection of galactose in urine (Fowweather, F. S.) 718
Tortoise, phosphoric esters in muscle of (Caldwell, P. C.) 458
Toxin production by *Corynebacterium diphtheriae*, mechanism of effect of iron and cobalt on (Clarke, W. A. & Clarke, G. D.) xvi
Translocation of betaine in plants (Cromwell, B. T. & Rennie, S. D.) 189
Trehalose, action of mould enzymes on (Bealing, F. J.) 93
Trialkylsulphonium chlorides, oxidation to sulphate in rat (Maw, G. A.) 42
Tricarballylic acid, chromatography of, estimation of (Isherwood, F. A. & Hanes, C. S.) 824
Tricarboxylic acid cycle in hepatopancreas of *Helix pomatia* (Rees, K. R.) 478
Tricarboxylic acid cycle in oxidation of succinate by *Pseudomonas fluorescens* (Kogut, M. & Podoski, E. P.) 800
Trilysine, and end product of enzymic hydrolysis (Waley, S. G. & Watson, J.) 328
Tri-*o*-cresyl phosphate, tocopherol deficiency produced by (Myers, D. K., Mulder, H. E. W. & Simons, E. G. L.) i
Triose phosphate, in oxidation of glycerol by mycobacteria (Hunter, G. J. E.) 320
Triphosphatase in muscle, relation to myoglobin (Lawrie, R. A.) 305
Triterpenoid alcohols and their derivatives, molecular rotations of (Klyne, W. & Stokes, W. M.) xxviii
Triticum vulgare Vill. var. Atle, coleoptiles, roots, glycinebetaine content of (Cromwell, B. T. & Rennie, S. D.) 189
Tropomyosin of rabbit muscle (Perry, S. V.) 114
Trypsin, action on polylysine (Waley, S. G. & Watson, J.) 328
Tryptophan, oxidation of, in pea-seedling tissues and extracts (Wiltshire, G. H.) 408
Tryptophan-tryptophanase action, possible model of (Baker, J. W.) xxxii
Tryptophan-tryptophanase reaction (Gooder, H. & Happold, F. C.) xxxii
Turbidimetric estimation of hyaluronate (Pearce, R. H.) 472
Turbidimetric estimation of hyaluronidase (Pearce, R. H.) 467
Tyrosinase in larvae of *Plusia gamma* (Goodwin, T. W.) 834
Tyrosine-rich substance in nodules from rheumatic fever patients (Consden, R., Glynn, L. E. & Stanier, W. M.) 248

Urea in degradation of elastin (Bowen, T. J.) 766
[^{14}C]Urea, metabolism in cats not secreting gastric juice (Kornberg, H. L., Davies, R. E. & Wood, D. R.) ii, iii
Urease, effect of pH on Michaelis constants (Dixon, M.) 162
Urine, amino acids and other ampholytes of (Westall, R. G.) 244
Urine, chemical estimation of sodium in, potassium as source of inaccuracy in (Aitken, E. H. & Preedy, J. R. K.) 211
Urine, detection of galactose in (Fowweather, F. S.) 718
Urine, determination of sodium and potassium in (Holiday, E. R. & Preedy, J. R. K.) 214
Urine, diazo reacting pigments of (Cole, P. G., Lathe, G. H. & Billing, B. H.) xiii
Urine, estimation of creatine in, application of diacetyl reaction to (Ennor, A. H. & Stocken, L. A.) 310
Urine, human, detection and identification of metabolites of *iso*nicotinic acid hydrazid in (Cuthbertson, W. F. J., Ireland, D. M. & Wolff, W.) 669
Urine, *p*-hydroxyphenylacetic acid (PHPA) in, significance of (Återman, K., Boscott, R. J. & Cooke, W. T.) xvii
Urine, separation of α-keto acids by paper chromatography (Smith, M. J. H. & Taylor, K. W.) xxx
Urines, acute porphyria, 'Waldenström' porphyrin of (Kennard, O. & Rimington, C.) 105; (Nicholas, R. E. H. & Rimington, C.) 109
Urines, excretion in, of phenolic acids in rat, effect of dietary deficiencies, aminopterin, ascorbic acid, chloretone and antibiotics (Boscott, R. J. & Greenberg, S. M.) xviii
Urines of bulls and pregnant cows, lipids from (Broadbent, I. E., Brooks, R. V. & Klyne, W.) vi
Uroporphyrin esters I and III, separation by paper chromatography (Falk, J. E. & Benson, A.) 101
Uroporphyrins from cases of cutaneous, acute and congenital porphyria, chromatographic study (Canivet, J. & Rimington, C.) 867

Valeric acids, α- and β-methylbutyric acids, occurrence in hair grease of dog (Brouwer, E. & Nijkamp, H. J.) 444
Valine, not a precursor of methyl groups (Arnstein, H. R. V. & Crawhall, J. C.) 280
Vibrio, production of keto acids from acetate by a (Dagley, S. & Patel, N. D.) xxxvi
Vicia faba L., cotyledons, shoots, roots, glycinebetaine content of (Cromwell, B. T. & Rennie, S. D.) 189
Virus receptor-substance, influenza, studies on (McCrea, J. F.) 132
Viruses, 5-hydroxymethylcytosine in deoxyribonucleic acid of (Wyatt, G. R. & Cohen, S. S.) 774
Vitamin A, studies in (Lowe, J. S. & Morton, R. A.) 681
Vitamin B_{12}-like compounds, combination with sow's whey and 'intrinsic factor' concentrates (Gregory, M. E. & Holdsworth, E. S.) 830, ix

'Waldenström' prophyrin, of acute porphyria urines (Kennard, O. & Rimington, C.) 105; (Nicholas, R. E. H. & Rimington, C.) 109
Water, active transport by kidney-cortex slices (Whittam, R. & Davies, R. E.) 880
Water in hepatic cells, effect of fasting (Harrison, M. F.) 204
Waxes in fish lipids (Lovern, J. A. & Olley, J.) 686
Whale, finner (*Balaenoptera physalus*), milk fats of, component acids of (Cama, J. S. & Meara, M. L.) 365
Whey, sow's milk, combination of vitamin B_{12}-like compounds with (Gregory, M. E. & Holdsworth, E. S.) 830, ix

Xanthine oxidase, effect of pH on Michaelis constants (Dixon, M.) 162
X-ray diffraction patterns, identification and classification of some porphyrins on (Kennard, O. & Rimington, C.) 105
X-ray spacings of α-hydroxy-*n*-fatty acids (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 707

Yeast, active transport of sodium and potassium ions in, effects of cortisone, deoxycorticosterone and other steroids on (Conway, E. J. & Hingerty, D.) 455
Yeast cerebrin, α-hydroxy-*n*-fatty acids of (Chibnall, A. C., Piper, S. H. & Williams, E. F.) 711
Yeast fermentation, nature of inhibition by D-glucosone (Johnstone, J. H. & Mitchell, I. L. S.) xvii

Zinc in hepatic cells, effect of fasting (Harrison, M. F.) 204
Zinc in human semen, spermatozoa and seminal plasma (Mawson, C. A. & Fischer, M. I.) 696
Zinc ions in aqueous solution, complexes of simple peptides with (Perkins, D. J.) xxviii

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **319th Meeting** *of the Biochemical Society was held in the Department of Biochemistry, University of Oxford, South Parks Road, Oxford, on Saturday, 20 June* **1953**, *at* **11** *a.m., when the following papers were read:*

COMMUNICATIONS

Tocopherol Deficiency Produced by Tri-*o*-cresyl Phosphate. By D. K. Myers, H. E. W. Mulder and Erica G. L. Simons. (*Pharmaco-Therapeutic Laboratory, University of Amsterdam, Amsterdam, Holland*)

Small amounts of tri-*o*-cresyl phosphate (TOCP) sufficient to inhibit the B-type ali-esterase (tributyrinase) of serum do not have any visible effect on rats. However, chronic administration of larger amounts of TOCP to male rats on a diet containing wheat-germ oil (cf. Mendel & Myers, 1952) gives rise to an irreversible sterility within 2–3 months and dystrophic paralysis of the hind legs about 10 months later. These effects might be caused directly by esterase inhibition but could also be due to tocopherol deficiency; an antagonism between tocopherol and TOCP has been suggested previously by Bloch & Hottinger (1943), Meunier, Vinet & Jouanetteau (1947) and Draper, James & Johnson (1952).

Intraperitoneal injection of 1 ml. TOCP/kg. causes an appreciable drop in the level of tocopherol in the serum of rats fed on a normal diet. Triphenyl phosphate, another ali-esterase inhibitor, has a similar effect. TOCP also prevents the normal increase in serum tocopherol after feeding 2 ml. of wheat-germ oil containing 0·2 % free tocopherol, or after feeding synthetic (±)-α-tocopherol in a large volume of arachis oil. However, the administration of TOCP does not affect the rate at which tocopherol is removed from the blood, nor does it prevent the tocopherol in the blood from penetrating into the tissues. The tocopherol deficiency produced by TOCP in animals on a normal diet is probably due to poor absorption of the tocopherol from the intestine; this deficiency can be prevented by feeding a highly concentrated solution of tocopherol, in which case the tocopherol is better absorbed.

These experiments suggested that it would be of interest to investigate the effect of tocopherol on the paralysis produced in chickens within two weeks after the administration of TOCP (cf. Earl & Thompson, 1952). The chickens were given divided doses of 50 mg. (±)-α-tocopherol/kg./day in a 17 % solution in oil, beginning 30 hr. before the TOCP was administered *per os*; the average serum concentration during the experiment was 4 mg. tocopherol/100 ml. as compared with a normal concentration of approximately 0·2 mg./100 ml. However, the supplement of tocopherol had no beneficial effects on the paralysis produced by TOCP.

REFERENCES

Bloch, H. & Hottinger, A. (1943). *Z. Vitaminforsch.* **13**, 9.

Draper, H. H., James, M. F. & Johnson, B. C. (1952). *Fed. Proc.* **11**, 204.

Earl, C. J. & Thompson, R. H. S. (1952). *Brit. J. Pharmacol.* **7**, 685.

Mendel, B. & Myers, D. K. (1952). *Nature, Lond.*, **170**, 928.

Meunier, P., Vinet, A. & Jouannetteau, J. (1947). *Bull. Soc. Chim. biol., Paris*, **29**, 507.

Androgenic Hormones and Ali-esterase Inhibition. By D. K. Myers, S. P. Simons and Erica G. L. Simons. (*Pharmaco-Therapeutic Laboratory, University of Amsterdam, Amsterdam, Holland*)

The stimulatory action of testosterone propionate on the growth of the seminal vesicle and prostate gland of immature rats can be reduced to about 30 % of normal by an ali-esterase inhibitor, tri-*o*-cresyl phosphate (TOCP); the action of testosterone is not significantly affected under the same conditions (Myers & Simons, 1953). Similar results are obtained when castrated rats are used, and when the rats are fed with extra (±)-α-tocopherol to counteract the tocopherol deficiency produced by large doses of TOCP. Moreover, the effect of TOCP can be duplicated by triphenyl phosphate (TPhP), an ali-esterase inhibitor with less toxicity than TOCP. These observations support the conclusion that

testosterone propionate does not exert its androgenic action before it has been hydrolysed in the tissues.

Analogous experiments were carried out using 10 i.u. of chorionic gonadotrophin ('Pregnyl', Organon) to stimulate the production of the natural androgenic hormone in the interstitial cells of the testis of immature rats. 15 μl. of TOCP injected subcutaneously in three divided doses cause complete inhibition of the ali-esterase (tributyrinase) activity of the interstitial cells of the testis and about 75 % inhibition of the tributyrinase activity of the seminal vesicle and prostate gland; the growth of these organs is not diminished. Larger amounts (150 μl.) of TOCP do antagonize the effect of gonadotrophin on the growth of the accessory glands; however, the growth of the testis is scarcely affected by TOCP in these experiments, while the stimulatory action of testosterone propionate on the tubuli of the testis could be completely abolished by TOCP or TPhP. Further experiments showed that the androgenic effects produced by gonadotrophin in hypophysectomized animals are not antagonized by TPhP in amounts (200 mg./100 g. body weight) which cause more than 90 % inhibition of the tributyrinase activity of the seminal vesicle and prostate gland.

These results do not permit any definite conclusion concerning the nature of the natural androgenic hormone (cf. Dingemanse, Freud & Polak, 1948). However, they do indicate that those aliesterases which are inhibited by TPhP and TOCP, are not essential for the synthesis or for the action of the natural androgenic hormone in rats.

REFERENCES

Dingemanse, E., Freud, J. & Polak, J. J. (1948). *Arch. int. Pharmacodyn.* **76**, 44.

Myers, D. K. & Simons, S. P. (1953). *Biochem. J.* **53**, xvii.

The Metabolism of [^{14}C]Urea in Cats not Secreting Gastric Juice. By H. L. Kornberg, R. E. Davies and D. R. Wood. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry; and Department of Pharmacology and Therapeutics, University of Sheffield*)

After intravenous injection of [^{14}C]urea into anaesthetized cats (15 experiments), whose ureters were tied to prevent loss of isotope in the urine, 0·2–0·3 %/hr. of the injected ^{14}C was expired as $^{14}CO_2$ (Kornberg & Davies, 1952). Calculations and experiments show that non-enzymic decomposition of urea accounted for less than one-hundredth of this breakdown and that enzymic cleavage of urea must occur. This cleavage was not due to urease in any of the abdominal viscera, since the removal of these organs before or during the experiments did not affect the rate of expiration of $^{14}CO_2$. Although, in all of six cats examined, aqueous suspensions of the gastric mucosae had urease activity ($Q_{urea} = 2$–34, average 12), this gastric urease played no part in the observed urea hydrolysis, because no fall in the rate of $^{14}CO_2$ expiration occurred on removal of the stomach during the experiments, or on clamping off the blood supply of the stomach. This suggested that in these cats gastric urease did not come into effective contact with circulating urea. However, when the blood flow was restored after clamping for 2 hr., a sharp rise in $^{14}CO_2$ output indicated that the prolonged anoxia of the stomach, by damaging the tissues (cf. Lutwak-Mann, 1947), had allowed the circulating urea to react with the gastric urease (see Kornberg, Davies & Wood, 1953).

The enzymic breakdown of urea was completely abolished by removal of the head and neck of the cats. No urease activity could be detected in suspensions of brain, tongue and muscle, but suspensions of oesophagus, and saline washings of the mouth and nasopharynx, contained sufficient urease to account for the observed *in vivo* urea breakdown. The enzymic breakdown of urea was abolished by treatment of cats for 4 days prior to injection of [^{14}C]urea with an antibacterial mixture of penicillin, terramycin and sulphaguanidine (suggested by Drs A. Baird Hastings and R. Dintzis, Harvard University). Since this mixture did not inhibit urease either *in vitro* or *in vivo*, but removed urease-containing organisms from the cats, it is concluded that the observed hydrolysis of [^{14}C]urea in cats not secreting gastric juice is due to the action of urease of bacterial origin, situated in the head and neck of the animals.

REFERENCES

Kornberg, H. L. & Davies, R. E. (1952). *2nd Int. Congr. Biochem. Abstr. Commun.* p. 144.
Kornberg, H. L., Davies, R. E. & Wood, D. R. (1953). (Next abstract.)
Lutwak-Mann, C. (1947). *Biochem. J.* **41**, 19.

The Metabolism of [^{14}C]Urea in Cats Secreting Gastric Juice. By H. L. KORNBERG, R. E. DAVIES and D. R. WOOD. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry; and Department of Pharmacology and Therapeutics, University of Sheffield*)

The *in vivo* activity of gastric urease, which could not be observed with cats not secreting gastric juice (Kornberg, Davies & Wood, 1953), became apparent during gastric secretion. After intravenous infusion of histamine or 2-benzyldihydroglyoxaline hydrochloride, the expiration of $^{14}CO_2$ by cats injected with [^{14}C]urea increased sharply, the rate of increase of specific activity being paralleled by the rate of secretion of juice. No such increase in $^{14}CO_2$ output occurred in gastrectomized cats similarly treated. In four cats, the rates of [^{14}C]urea hydrolysis through the action of gastric urease, calculated from the amounts of $^{14}CO_2$ expired, were 0·011, 0·012, 0·023 and 0·023 m-mole/hr.; when calculated from the ammonia contents of the secreted juice (cf. Von Korff, Ferguson & Glick, 1951), these rates were 0·019, 0·012, 0·022 and 0·023 m-mole/hr. respectively. These rates were less than one-fiftieth of the concomitant rates of acid secretion.

This *in vivo* activity of gastric urease also appeared when, after injections of histamine and urogastrone, a non-acid juice was secreted. No activity was elicited by varying the blood flow through the stomach or by filling the stomach with 0·1N-HCl. When [^{14}C]urea solution (pH 1·5 or 6·8) was put into the lumen of the stomach and maintained at various pressures, $^{14}CO_2$ of high specific activity was expired, although the specific activity of blood urea remained very low. The rate of hydrolysis of urea varied with the rate of flow of liquid forced through the mucosa.

Even when secreting gastric juice, cats pretreated with penicillin, terramycin and sulphaguanidine (Kornberg *et al.* 1953) did not break down urea, and aqueous suspensions of their gastric mucosae were entirely devoid of urease activity. Whereas the juice secreted by untreated cats contained 0·51–0·67 mM urea (average 0·60 mM) and 3·6–4·4 mM ammonia (average 4·2 mM) (cf. Teorell, 1933), the gastric juice of treated cats contained 2·6–3·4 mM urea (average 3·0 mM) and no detectable ammonia. Since the concentration of urea in the blood was approx. 7 mM, this showed that there is a resistance to the diffusion of urea into the gastric juice.

These results show that gastric urease is of bacterial origin, and that its activity depends on the rate of passage of urea-containing liquid across the mucosa. They imply that urease plays no essential role in the physiology of the stomach.

REFERENCES

Kornberg, H. L., Davies, R. E. & Wood, D. R. (1953). (Previous abstract.)

Teorell, T. (1933). *Scand. Arch. Physiol.* **66**, 225.

Von Korff, R. W., Ferguson, D. J. & Glick, D. (1951). *Amer. J. Physiol.* **165**, 695.

The Action of Monofluoroacetate on the Metabolism of *Rhodospirillum rubrum*. By S. R. ELSDEN and J. G. ORMEROD. (*A.R.C. Unit of Microbiology, Department of Microbiology, University of Sheffield*)

Liébecq & Peters (1949) have shown that citrate accumulates in animal tissues poisoned with monofluoroacetate. Peters, Wakelin & Buffa (1953) have provided evidence that the drug is converted to a fluorine-containing tricarboxylic acid, which is the actual inhibitor. The drug is thus a useful aid to the study of the tricarboxylic acid cycle.

The action of monofluoroacetate on the light and dark metabolism of the photosynthetic organism *Rhodospirillum rubrum* has been investigated. At a concentration of $8·3 \times 10^{-4}$ M, monofluoroacetate inhibited strongly the photometabolism of acetate, *n*-butyrate, pyruvate, oxaloacetate and hydrogen, but had only a slight effect when propionate, DL-lactate, succinate, fumarate and DL-malate were the substrates. In contrast, the dark oxidation of acetate, propionate, *n*-butyrate, succinate, fumarate and pyruvate was strongly inhibited by monofluoroacetate at a concentration of $8·3 \times 10^{-4}$ M.

Citrate was found to accumulate in the presence of the drug when the organism photosynthesized on pyruvate or oxaloacetate; but only traces were found when acetate, succinate, fumarate, or a mixture of hydrogen and carbon dioxide were the substrates under these conditions.

In the presence of monofluoroacetate, citrate accumulated during the dark oxidation of succinate and pyruvate.

REFERENCES

Liébecq, W. D. & Peters, R. A. (1949). *Biochim. Biophys. Acta, Amst.*, **3**. 215.

Peters, R. A., Wakelin, R. W. & Buffa, P. (1953). *Proc. Roy. Soc.* B, **140**, 497.

Purification and some Properties of Aconitase. By J. F. MORRISON. (*Department of Biochemistry, University of Oxford*)

The activation of aconitase by Fe^{2+} and cysteine, as shown by Dickman & Cloutier (1950, 1951), has been confirmed using a highly purified solution of the enzyme. Aconitase was purified 24-fold by low-temperature alcohol and ammonium sulphate fractionation. Electrophoretic analysis of the final preparation showed that the main component, which was responsible for enzyme activity, formed 75–80% of the total protein. Three other small components were also present. When the main component was separated it was found to be free of pigment and capable of converting both *cis*-aconitate and *iso*citrate to citrate.

Both Fe^{2+} and cysteine were required for maximum enzyme activity. There was only slight activity in the absence of these agents, or in the presence of each separately. The degree of activation of the enzyme increased as the fractionation proceeded, being most marked after dialysis. Maximum enzyme activity was attained only after incubation with Fe^{2+} and cysteine for 30–60 min. at 0° or 3–4 min. at 30°. No activation occurred if the solutions were shaken in air during the activation period.

Some of the properties of the enzyme are of interest. The activated enzyme was rapidly inactivated by phosphate in the absence of substrate, but there was no inactivation and even apparent activation when phosphate and substrate were added simultaneously. Similar results were obtained with glycerophosphate, *N*-ethyl morpholine and veronal acetate buffers at pH 7·4. Aconitase was very sensitive to pH values below 5·0, complete loss of activity occuring at pH 4·0. The enzyme was reasonably stable to heat. No loss of activity was found until a temperature of 51° was attained, after which denaturation was rapid.

REFERENCES

Dickman, S. R. & Cloutier, A. A. (1950). *Arch. Biochem.* **25**, 229.

Dickman, S. R. & Cloutier, A. A. (1951). *J. biol. Chem.* **188**, 379.

Porphyrin Synthesis by *Rhodopseudomonas spheroides*. By JUNE LASCELLES. (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

It is now well established by studies with isotopically labelled compounds that glycine and intermediates of the tricarboxylic acid cycle are precursors of porphyrins in animals (Shemin & Kumin, 1952; Muir & Neuberger, 1950). Glycine is also a precursor of the porphyrins excreted by *Corynebacterium diphtheriae* (Hale, Rawlinson, Gray, Holt, Rimington & Wilson Smith, 1950). The mechanisms involved are largely unknown.

Porphyrin synthesis has been studied with cell suspensions of *Rhodopseudomonas spheroides*, a member of the Athiorhodaceae. The cells were grown anaerobically in the light on salts, malate, glutamate and B-group vitamins, and under these conditions contained bacteriochlorophyll and carotenoids. When incubated anaerobically in the light in a mixture containing salts, glycine, α-ketoglutarate and fumarate, they formed large amounts of porphyrins (200–300 μg./mg. dry wt. of cells in 24 hr.). These appeared in the supernatant fluid, only traces occurring in the cells. The porphyrins were estimated spectrophotometrically at 403 mμ., using coproporphyrin type III as standard. They consisted mainly of this compound with small amounts of uroporphyrin type I and other porphyrins.* Under the usual experimental conditions there was a twofold increase in bacteriochlorophyll in the cells.

Omission of glycine or α-ketoglutarate from the system reduced porphyrin synthesis to about one-tenth; fumarate had a less marked effect. Mg^{2+} was essential; omission of Mn^{2+} reduced synthesis to about one-tenth, and of NH_4^+ to about one-fifth. No synthesis occurred when the cells were incubated either anaerobically or aerobically in the dark; the organism can grow under the latter conditions but forms neither bacteriochlorophyll nor carotenoids.

Iron (5×10^{-6} M or more) added as ferric citrate or ferrous ammonium sulphate almost completely prevented the appearance of porphyrins, but it increased bacteriochlorophyll formation threefold. There appears to be no simple relationship between the decrease in porphyrins and the increase in bacteriochlorophyll.

* I am indebted to Dr J. E. Falk for the chromatographic analyses.

Formation of large amounts of porphyrins by a photosynthetic organism has also been observed with mutant strains of *Chlorella*, unable to synthesize chlorophyll (Bogorad & Granick, 1952).

REFERENCES

Bogorad, L. & Granick, S. (1952). *Nature, Lond.*, **170**, 321.

Hale, J. H., Rawlinson, W. A., Gray, C. H., Holt, L. B., Rimington, C. & Wilson Smith (1950). *Brit. J. exp. Path.* **31**, 96.

Muir, H. M. & Neuberger, A. (1950). *Biochem. J.* **47**, 97.

Shemin, D. & Kumin, S. (1952). *J. biol. Chem.* **198**, 827.

The Biosynthesis of Penicillin. By H. R. V. Arnstein and P. T. Grant. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

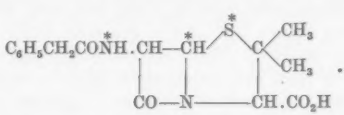
Although much is known about the biosynthesis of the penicillin side-chain, the origin of the fused thiazolidine-β-lactam rings, which form the main part of the molecule, has so far not been elucidated (see, for example, Behrens, 1949).

In the present work, the biosynthesis of this portion of the penicillin molecule has been investigated using amino acids labelled with ^{14}C, ^{15}N or ^{35}S. The labelled compounds were added to growing submerged cultures of *Penicillium chrysogenum* WIS 48–701, and at the end of the fermentation (usually 6 days) the amount of isotope incorporated into penicillin was measured. In most cases the penicillin was then degraded chemically in order to locate the position of the labelled atoms.

When [α-^{14}C]glycine, DL-[γ-^{14}C]valine, DL-[β-^{14}C]-serine and DL-[β-^{14}C]cystine were used in comparable amounts (0·4 μmole/ml. broth/day), it was found that the molar radioactivity of the penicillin was approximately 7·2, 17·3, 19·6 and 32·2 % of that of the administered glycine, valine, serine and cystine (calculated as cysteine) respectively. Degradation indicated that valine was incorporated exclusively into the penicillamine moiety of the molecule, whereas glycine and cystine gave rise to the β-lactam carbon atoms. Since cystine was found to be the best precursor further experiments were carried out with this amino acid.

Using L- and D-[β-^{14}C]cystine it was found that the incorporation of L-cystine into penicillin was about 5× that of the D-enantiomorph, suggesting that a stereochemically specific reaction is involved. The utilization of the complete cystine molecule is further demonstrated by the conversion of L-[^{35}S, ^{15}N, β-^{14}C]cystine into penicillin with similar dilution of the S, N and C isotopes (3·4, 4·1 and 3·8 respectively). Degradation of the penicillin showed that about 95 % of the ^{14}C was located at the carbon atom common to the thiazolidine and β-lactam rings. No ^{15}N was found in the penicillamine nitrogen.

The position of the labelled atoms in the penicillin molecule is shown (*) in the following formula:



The significance of these results and possible mechanisms of penicillin biosynthesis from cystine will be discussed.

REFERENCE

Behrens, O. K. (1949). *The Chemistry of Penicillin*, p. 657. Edited by Clarke, H. T., Johnson, J. R. and Robinson, Sir Robert. Princeton: Princeton University Press.

Acetylcholine Metabolism in Electrically Stimulated Slices of Cerebral Cortex. By E. V. Rowsell, Beit Memorial Research Fellow. (*Biochemical Laboratories, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, S.E.* 5)

Acetylcholine in brain slices exists bound as a pharmacologically inactive complex, and non-hydrolysable in that combination despite the very great choline esterase (ChE) activity of brain. This complex can dissociate to liberate the freely diffusible, active and ChE-labile acetylcholine (Mann, Tennenbaum & Quastel, 1938). Electrical stimulation and convulsions diminish considerably the total extractable acetylcholine of brain *in vivo* (Richter & Crossland, 1949). The hypothesis was considered that, associated with electrical activity in the brain, bound acetylcholine is liberated as the active dif-

fusible form, which is then quickly hydrolysed under the influence of ChE. The techniques developed by McIlwain (1951) for the electrical stimulation of tissue slices were employed to test this hypothesis.

Slices of guinea-pig cerebral cortex were allowed to respire, each in 5 ml. of Krebs-phosphate-glucose medium so that respiration could be observed. Eserine was present to inhibit ChE. After 60–75 min. aerobic incubation at 37° some slices were electrically stimulated for 15–20 min. The acetylcholine content of each medium was assayed using eserinized frog rectus abdominis muscle. In a series of seventeen such experiments, fourteen gave an acetylcholine level in the medium more than 40 % greater where the slices had been stimulated. The mean for the media of unstimulated slices was $2{\cdot}38 \pm 0{\cdot}32$ μg. ACh/g. brain slice, and $3{\cdot}50 \pm 0{\cdot}28$ for the stimulated slices.

The bound acetylcholine of cerebral cortex slices was also assayed. 95–115 min. incubations without eserine were followed by 30 min. stimulation to some slices. In seven such experiments six gave more than 25 % decrease in bound acetylcholine on stimulation. The mean for the unstimulated slices was $4{\cdot}91 \pm 0{\cdot}38$ μg. ACh/g. brain slice, and $3{\cdot}49 \pm 0{\cdot}25$ for the stimulated slices.

It seems that electrical stimulation induces a dissociation of the bound complex, allowing the liberation of free acetylcholine. The formation of acetylcholine *de novo* as an additional consequence of electrical stimulation is a possibility not excluded.

REFERENCES

McIlwain, H. (1951). *Biochem. J.* **49**, 382.
Mann, P. J. G., Tennenbaum, M. and Quastel, J. H. (1938). *Biochem. J.* **32**, 243.
Richter, D. & Crossland, J. (1949). *Amer. J. Physiol.* **159**, 247.

Hippuric Acid Formation in the Locust. By L. Friedler and J. N. Smith. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

It has been shown that the locust can acetylate the aromatic amino group in sulphadimidine (Myers & Smith, 1953) and can conjugate *m*-aminophenol to form β-*m*-aminophenylglucoside (Myers & Smith, unpublished data). We have now been able to show that the locust can form hippuric acids from aromatic acids. Locusts (*Locusta migratoria migratorioides*) were fed or injected with benzoic, *p*-aminobenzoic, *p*-nitrobenzoic and salicylic acids. An examination of extracts of the excreta by paper chromatography gave evidence of the formation of hippuric and *p*-aminohippuric acids from benzoic and *p*-aminobenzoic acids. Hippuric acid, m.p. and mixed m.p. 186°, was isolated from faeces of locusts feeding on biscuit soaked in benzoic acid solution. *p*-Nitrobenzoic acid was reduced to a considerable extent and excreted as *p*-aminobenzoic and *p*-aminohippuric acids which were detected chromatographically in the excreta. No metabolites of salicylic acid were identified and the unchanged acid was detected in the faeces.

Quantitative measurements showed that about 70 % of a 1 mg. dose of *p*-aminobenzoic acid was excreted in 3 days, 10–22 % of the dose being present as the glycine conjugate.

REFERENCE

Myers, C. M. & Smith, J. N. (1953). *Biochem. J.* **54**, 276.

Some Lipid Fractions from the Urines of Bulls and Pregnant Cows. By Irene E. Broadbent, R. V. Brooks and W. Klyne. (*Postgraduate Medical School, London W.* 12)

Lipids from the urines of bulls and pregnant cows have been studied both as water-soluble conjugates and in the free state after hydrolysis. No steroids have been isolated in the present work, although Marker and his colleagues isolated several steroids from bulls' and cows' urine (Marker, 1938, 1939; Marker, Wittle & Lawson, 1938).

Conjugated lipids were extracted from the urine with butanol and fractionated by the methods of Klyne, Schachter & Marrian (1948). The glucuronide and sulphate contents of the fractions were determined (Bray, Humphris, Thorpe, White & Wood, 1952). The conjugate fraction from bulls' urine containing the more sparingly water-soluble potas-

sium salts yielded a solid which was purified via the *p*-toluidine salts (cf. Barton & Young, 1943); it appears to be a mixture of the sulphates of two closely related phenols of empirical formulae approximately $C_9H_{12}O_2$.

Other samples of urine were hydrolysed with acid and separated into acidic, phenolic and neutral fractions in the usual way. The neutral fraction was treated with phthalic anhydride to give alcoholic and non-alcoholic fractions, and the alcohols further divided into ketones and non-ketones with Girard's reagent.

The non-ketonic fractions were submitted to separation with digitonin. The 'β' fractions (precipitated by digitonin), which were very small, were examined by chromatography as benzoates on alumina. This method, which had been successful in separating steroids from similar fractions of pregnant mares' urine (Brooks, Klyne, Miller & Paterson, 1952) failed to give any solid products.

Chromatography of the non-ketonic 'α' fractions (not precipitated by digitonin) has yielded much 5-hydroxy-*cis*-tetrahydro-ionol (4-3′-hydroxybutyl-3:3:5-trimethyl*cyclo*hexanol) from both the bull and pregnant cow. Prelog, Führer, Hagenbach & Schneider (1948) previously isolated this compound (diol A) from the urine of pregnant mares (cf. Prelog & Osgan, 1952).

The ketonic alcohol fraction from bulls' urine was distilled at 90–150°/1 mm. and the non-volatile material separated with digitonin. The fractions so obtained have been examined.

We are indebted to the Agricultural Research Council for expenses and for a personal grant to one of us (R. V. B). We are grateful to Dr J. A. Laing, Veterinary Laboratory, University of Bristol, for urine of bulls and cows.

REFERENCES

Barton, A. D. & Young, L. (1943). *J. Amer. chem. Soc.* **65**, 294.
Bray, H. G., Humphris, B. G. Thorpe, W. V., White, K. & Wood, P. B. (1952). *Biochem. J.* **52**, 412.
Brooks, R. V., Klyne, W., Miller, E. & Paterson, J. Y. F. (1952). *Biochem. J.* **51**, 694.
Klyne, W., Schachter, B. & Marrian, G. F. (1948). *Biochem. J.* **43**, 231.
Marker, R. E. (1938). *J. Amer. chem. Soc.* **60**, 2442.
Marker, R. E. (1939). *J. Amer. chem. Soc.* **61**, 944.
Marker, R. E., Wittle, E. L. & Lawson, E. J. (1938). *J. Amer. chem. Soc.* **60**, 2931.
Prelog, V., Führer, J., Hagenbach, R. & Schneider, R. (1948). *Helv. chim. Acta*, **31**, 1799.
Prelog, V. & Osgan, M. (1952). *Helv. chim. Acta*, **35**, 986.

The Preparation and Properties of DL-Cysteinol and some of its Derivatives. By J. C. Crawhall and D. F. Elliott. (*National Institute for Medical Research, Mill Hill, London, N.W.* 7)

Among the methods recently proposed for identifying the carboxyl end groups of proteins is that of reducing the carboxyl groups with a metallohydride (Fromageot, Jutisz, Meyer & Penasse, 1951; Chibnall & Rees, 1951). After total acid hydrolysis the amino acid from the carboxyl end of the protein is liberated as the corresponding amino alcohol. Most of the amino alcohols have been characterized but the preparation of cysteinol (2-amino-3-thiolpropanol) has not so far been reported. There has been no report of attempts to reduce cysteine methyl ester with either metallic sodium or a metallohydride and it may be that the free thiol group interferes with the reduction. The free thiol and amino groups of cysteine methyl ester hydrochloride can be masked by condensation with ethyl benzimidate (Crawhall and Elliott, 1951) to give 4-carbomethoxy-2-phenyl thiazoline. This ester has now been reduced with lithium aluminium hydride in tetrahydrofuran to 4-hydroxymethyl-2-phenyl thiazoline, which is a crystalline solid with m.p. 71°. On acid hydrolysis this thiazoline gives a high yield of cysteinol and benzoic acid which are easily separated. For convenience in isolation the cysteinol can be oxidized in a stream of air to cystinol which gives a stable crystalline hydrochloride with m.p. 183°.

The identification of cysteine as the residue at the carboxyl end of a protein would be complicated during isolation procedure by the aerial oxidation of the cysteinol in the presence of cysteine residues from other parts of the protein chain. A basic amino acid containing one molecule of cysteinol and one molecule of cysteine, joined through an S—S bridge, could be formed. This difficulty could be overcome by oxidizing all the —SH groups to $-SO_3H$ groups. The R_F values of cysteinol and its derivatives have been compared in various solvents with similar derivatives in the cysteine series.

REFERENCES

Chibnall, A. C. & Rees, M. W. (1951). *Biochem. J.* **48**, xlvii.
Crawhall, J. C. & Elliott, D. F. (1951). *J. Soc. chem.* p. 2071.
Fromageot, C., Jutisz, M., Meyer, D. & Penasse, L. (1951). *Biochim. Biophys. Acta, Amst.*, **6**, 283.

Protein Synthesis in the Rat Brain. By M. K. GAITONDE and D. RICHTER. (*Neuropsychiatric Research Centre, Cardiff*)

Measurements were made of the rate of incorporation of methionine labelled with ^{35}S into the proteins of the rat brain. The methionine, which was administered by intracisternal injection, was the naturally occurring isomer prepared from yeast by the method of Williams & Dawson (1952). Control experiments showed that the radioactivity of the protein fraction of the brain was attributable mainly to methionine, with a smaller proportion of cysteine which was also combined in peptide linkages.

Figures for the specific activity ($^{35}S/^{32}S$) of the protein fraction at different times after injection showed that methionine injected *in vivo* passes rapidly into the proteins of the rat brain. The specific activity ratio Sp.A. protein S/Sp.A. acid-soluble S, which is independent of the amount of radioactive isotope injected, gives a satisfactory index of the rate of protein synthesis. Measurements in seven animals at 3 hr. after injection gave consistent figures with a mean of 1·23, S.D. 0·04, for this ratio for the brain and a mean of 0·28, S.D. 0·05, for the liver. These figures indicate that the rate of protein synthesis per gram tissue *in vivo* is relatively high in the rat brain.

REFERENCE

Williams, R. B. & Dawson, R. M. C. (1952). *Biochem. J.* **51**, 314.

The Effect of Growth on a Medium containing Methionine on the Synthesis of this Amino Acid by *Bacterium coli*. By S. WIJESUNDERA and D. D. WOODS. (*Department of Biochemistry, University of Oxford*)

The study of the synthesis of methionine by cell suspensions of *Bact. coli* incubated with homocysteine, serine, glucose, *p*-aminobenzoic acid and vitamin B_{12} (Gibson & Woods, 1952; Gibson, 1952) was extended to other strains. With one mutant (Y 44), requiring *p*-aminobenzoic acid for growth, synthesis was almost nil when the cells were harvested from the medium used to obtain cells deficient in *p*-aminobenzoic acid; i.e. basal medium supplemented with casein hydrolysate, purines and thymine. Cells from the basal medium plus *p*-aminobenzoic acid synthesized well. The effect was traced to a constituent of the casein hydrolysate, methionine, whose addition (10^{-3} M) to the basal medium plus *p*-aminobenzoic acid led to 'inactive' cells. Cohn, Cohen & Monod (1953) have recently reported very similar results. Under similar conditions the synthetic ability of ten other strains of *Bact. coli* was reduced to 10–30 % of the control value by the presence of 10^{-3} M DL-methionine in the growth medium.

A more detailed study was made with strain Y 44. The lowest concentration of DL-methionine giving 'inactive' cells was 10^{-4} M; L-cysteine, L-cystathionine and DL-homocysteine (all 10^{-3} to 10^{-4} M) had no action. Nineteen other DL-amino acids including leucine (tested singly at 10^{-3} M) reduced synthesis only by 0–15 %.

'Inactive' cells were not reactivated by the addition to the test system of extracts of 'active' cells or of yeast, or by 'Leucovorin' or pteroylglutamate. 'Inactive cells' did not appreciably destroy methionine, neither did extracts from them inhibit methionine synthesis by 'active' cells or affect the growth of the assay organism (*Leuconostoc mesenteroides* P 60).

Ability to synthesize methionine was lost or regained by successive single subcultures on media with and without methionine respectively. Incubation of harvested cells simply in buffered glucose with or without methionine did not change their synthetic ability. It appears that growth in the presence of methionine is required to induce the change in enzymic constitution. In a typical experiment in which synthesis was reduced to 6 % of the control, there was no significant difference in the viable count of the cells when plated on agar medium with and without methionine; it is unlikely therefore that a mutant unable to synthesize methionine was selected.

REFERENCES

Cohn, M., Cohen, G. & Monod, J. (1953). *C. R. Acad. Sci., Paris*, **236**, 746.

Gibson, F. (1952). *2nd Int. Congr. Biochem., Abstr.*, p. 80.

Gibson, F. & Woods, D. D. (1952). *Biochem. J.* **51**, v.

The Fate of ^{35}S-Labelled Arylsulphuric Acids after their Administration to the Rat. By JOYCE B. HAWKINS and L. YOUNG. (*Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E.* 1)

Although it is known that arylsulphatase occurs in animal tissues (Neuberg & Simon, 1925; Rosenfeld, 1925), various workers (e.g. Garton & Williams, 1949) have concluded that when the salt of an arylsulphuric acid such as potassium phenylsulphate, $C_6H_5OSO_3K$, is administered to an animal, almost all the compound is excreted unchanged in the urine. This conclusion has been based on the results of experiments using ordinary analytical methods, and it is not to be expected that these would reveal a small amount of hydrolysis of an administered arylsulphate. For this reason the question of whether these compounds are hydrolysed in the animal body has now been investigated by studying the fates of the potassium salts of ^{35}S-labelled arylsulphuric acids (phenylsulphuric acid, 1- and 2-naphthylsulphuric acid) following their administration to rats by stomach tube and by intraperitoneal injection.

The arylsulphates were synthesized using ^{35}S-labelled radioactive chlorosulphonic acid and were recrystallized until they showed constant specific activity and were free from inorganic sulphate. Each rat of a pair was given a dose of an aqueous solution of 0·05 m-mole of the potassium salt of the radioactive arylsulphuric acid. The pair of rats was housed in a metabolism cage and the urine excreted during the following 4 days was collected at intervals and analysed for its content of inorganic sulphate-^{35}S and total sulphate-^{35}S. When the compounds were given by intraperitoneal injection the amounts of ^{35}S excreted in the urine as inorganic sulphate corresponded to the following percentages of the ^{35}S administered as arylsulphate: phenylsulphate, 4; 1-naphthylsulphate, 11; 2-naphthylsulphate, 8. The results obtained after the compounds had been given by stomach tube were 5, 12 and 8 %, respectively. In every case the greater part of the arylsulphate was excreted unchanged in the urine, most of it within a few hours of dosing. Experiment showed that the arylsulphates studied did not undergo a significant amount of hydrolysis in the urine under the conditions used in the investigation, and it is concluded that a small but definite amount of hydrolysis of these compounds occurred within the organism. As essentially similar results were obtained whether an arylsulphate was given by stomach tube or by intraperitoneal injection, it appears that no significant hydrolysis of these compounds occurred in the gastro-intestinal tract.

REFERENCES

Garton, G. A. & Williams, R. T. (1949). *Biochem. J.* **45**, 158.

Neuberg, C. & Simon, E. (1925). *Biochem. Z.* **156**, 365.

Rosenfeld, L. (1925). *Biochem. Z.* **157**, 435.

The Combination of some Vitamin B_{12}-like Compounds with Sow's Whey and 'Intrinsic Factor' Concentrates. By MARGARET E. GREGORY and E. S. HOLDSWORTH. (*National Institute for Research in Dairying, University of Reading*)

Compounds are known to exist which are not simple derivatives of vitamin B_{12}, but which have 'vitamin B_{12}-activity' for micro-organisms: e.g. factors A and B (Ford and Porter, 1952) and pseudo-vitamin B_{12} (Pfiffner, Calkins, Peterson, Bird, McGlohen & Stipek, 1951). The microbiological inactivation of these vitamin B_{12}-like compounds, by an 'intrinsic factor' concentrate (prepared from desiccated hog stomach) and a concentrate from sow's whey, has been investigated. Both concentrates were prepared by $(NH_4)_2SO_4$ fractionation (Gregory, Ford & Kon, 1952).

The vitamin B_{12}-like compounds were purified by ionophoresis (Holdsworth, 1953) and equimolar solutions prepared by measuring the optical density at 367 mμ. in the presence of 0·01 % KCN and making the assumption that the $E^{1\%}_{1\,cm.}$ was the same for all these substances under these conditions. Measured by the *Escherichia coli* tube assay (Burkholder, 1951), factor A had 66 %, factor B 22 % and pseudo-vitamin B_{12} 8 % of the growth activity of a solution of vitamin B_{12} of the same optical density.

The vitamin B_{12}-binding activity of the sow's whey or 'intrinsic factor' concentrates was measured using the *E. coli* tube assay. Decreasing amounts of the concentrates were added to a fixed amount of vitamin B_{12}. Where the amount of 'binding material' in the tube completely 'bound' all the vitamin B_{12}, the assay organism was unable to grow. Where the quantity of 'binding material' was only sufficient to inactivate a part of the vitamin B_{12}, the assay organism responded to the residual 'unbound' vitamin which was then measured quantitatively by reference to a vitamin B_{12}

standard. From this, the amount of the vitamin 'bound' by the concentrate was calculated. The amounts of factors A and B and pseudo-vitamin B_{12} 'bound' were calculated in the same way, in each case referring to a standard of the particular substance under test.

Both concentrates 'bound' factors A and B and pseudo-vitamin B_{12} to the same extent as vitamin B_{12}. If, therefore, combination with intrinsic factor is important for the absorption of vitamin B_{12} or serves to protect it from assimilation by the intestinal flora, then the same process would apply to the other vitamin B_{12}-like compounds tested, since they are all 'bound' to the same extent.

REFERENCES

Burkholder, P. R. (1951). *Science*, **114**, 459.
Ford, J. E. & Porter, J. W. G. (1952). *Biochem. J.* **51**, v.
Gregory, M. E., Ford, J. E. & Kon, S. K. (1952). *Biochem. J.* **51**, xxix.
Holdsworth, E. S. (1953). *Nature, Lond.*, **171**, 148c.
Pfiffner, J. J , Calkins, D. J., Peterson, R. G., Bird, O. D., McGlohen, V. & Stipek, R. W. (1951). *Abstr. Pap. Amer. chem. Soc.* 120*th Mtg*, p. 22c.

Reversal of the Antibacterial Activity of Polymyxin B by Non-toxic Divalent Cations. By B. A. Newton. (*M.R.C. Unit for Chemical Microbiology, Biochemical Laboratory, University of Cambridge*)

Previous studies have shown that the addition of polymyxin to washed cell suspensions of *Pseudomonas aeruginosa* caused a release of pentose, phosphate and materials which had an absorption maxima at 260 mμ. (Newton, 1953). The leakage was maximum in the presence of concentrations of polymyxin which killed 99 % of the cells, higher concentrations inhibiting the leakage.

When washed cells were treated with polymyxin in the presence of magnesium it was found that the leakage of soluble constituents from the cells did not occur. This 'protective' action of magnesium could be reversed by the addition of 2 molecules of citrate/atom of magnesium present.

Growth experiments showed that the limiting inhibitory concentration of polymyxin could be increased by as much as 30 × by the addition of magnesium.

When suspensions of washed cells (containing about 2×10^9 viable cells/ml.) were treated with 15 μg. polymyxin/ml. for 5 min. at 30° less than 10 % of the cells remained viable. In the presence of M/400 magnesium 70–90 % of the cells were viable. Similar results have been obtained with other non-toxic divalent cations, e.g. Ca^{2+}, Mn^{2+} and Fe^{2+}.

A method for the photometric estimation of polymyxin has been developed, based on the modfied ninhydrin method of Troll & Cannan (1953). Using this method it has been found that washed cells absorb large amounts of polymyxin; the presence of divalent cations prevents this absorption.

There seem to be two possible explanations of the above results: (1) a competitive absorption between polymyxin and the cations for sites on or in the cells; (2) a formation of a polymyxin-metal ion complex with the resultant inactivation of the antibiotic. In view of the high cation/polymyxin ratio (*c.* 400 ions/polymyxin molecule) required to reverse the antibacterial activity the former hypothesis would seem to be more reasonable.

REFERENCES

Newton, B. A. (1953). *J. gen. Microbiol.* **8**, vi.
Troll, W. & Cannan, R. K. (1953). *J. biol. Chem.* **200**, 803.

Phosphorylation Coupled with Anaerobic Breakdown of Citrulline. By V. A. Knivett. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Sheffield*)

Cell-free extracts of *Streptococcus faecalis* convert citrulline to ornithine, ammonia and carbon dioxide (Akamatsu & Sekine, 1951), a reaction which is accompanied by phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) (Knivett, 1952). This process has been studied quantitatively with the isotopic and chromatographic techniques of Krebs, Ruffo, Johnson, Eggleston & Hems (1953), Eggleston & Hems (1952), and Krebs & Hems (1953).

The cell-free extract (Knivett, 1953) was incubated with inorganic phosphate containing

$^{32}PO_4$, magnesium ions, citrulline, succinate buffer and either ATP or ADP. Samples were analysed for inorganic phosphate and adenosine mono-, di- and tri-phosphates and the radioactivity of each component was estimated. The use of $^{32}PO_4$ makes it possible to distinguish between ATP formed by the action of myokinase (adenylate kinase) and by synthesis from ADP and inorganic phosphate.

Controls without citrulline showed that myokinase, and ATPase, but no ADPase were present. In the first 10 min. of incubation of the mixture containing ADP and citrulline, CO_2 was released, inorganic phosphate and ADP disappeared, ATP was formed, the four changes being approximately equivalent. Some adenosine monophosphate appeared within the first 10 min. but later partly disappeared. There was little change in the concentrations of the various phosphates after 10 min. although CO_2 continued to be released.

The ratio $\frac{\text{spec. act. of ATP}}{\text{spec. act. of inorganic phosphate}}$ (spec. act. expressed as cts./min./mole) increased from 0·42 after 1 min. to 1·23 after 90 min. This indicates that some ATP was formed by myokinase activity, and also that the isotope was present in both of the labile phosphates of ATP.

In other samples to which an ATP preparation, contaminated by about 20 % ADP, was added, the rate of CO_2 release was slower, with no appreciable change in the relative amounts of adenosine phosphates. A fall in specific activity of inorganic phosphate accompanied the incorporation of ^{32}P into the labile phosphate fraction. After 1 hr. 20 % of the original activity was in ATP and 2 % in ADP.

The author is grateful to Mr R. Hems for technical assistance and to Professor H. A. Krebs, F.R.S., and to the Director, Chemical Research Laboratory, Teddington, for permission to undertake this investigation.

REFERENCES

Akamatsu, S. & Sekine, T. (1951). *J. Biochem., Tokyo*, **38**, 349.

Eggleston, L. V. & Hems, R. (1952). *Biochem. J.* **52**, 156.

Knivett, V. A. (1952). *2nd Int. Congr. Biochem., Paris*, p. 86.

Knivett, V. A. (1953). *J. gen. Microbiol.* **8**, v.

Krebs, H. A., & Hems, R. (1953). *Biochim. et Biophys. Acta, Amst.* (in the Press).

Krebs, H. A., Ruffo, A., Johnson, M., Eggleston, L. V. & Hems, R. (1953). *Biochem. J.* **54**, 107.

Amino Acid Incorporation by Fragmented Staphylococcal Cells. By E. F. Gale and Joan P. Folkes. (*Medical Research Council Unit for Chemical Microbiology, Cambridge*)

Thick washed suspensions of *Staph. aureus* have been subjected to supersonic vibration in a buffered sucrose solution, and the resulting material fractionated on the centrifuge. A fraction P, consisting of partially disrupted cells, has been obtained which will incorporate ^{14}C-labelled glutamic acid if incubated in the presence of hexose diphosphate and adenosine triphosphate as energy source. When glutamic acid is the only amino acid present, incorporation ceases when the protein of fraction P has attained a radioactivity which depends upon the concentration of ^{14}C-glutamic acid in the incubation mixture. Fraction P labelled in this way will subsequently lose radioactivity if incubated with ^{12}C-glutamic acid and energy source but not if incubated with ^{12}C-glutamic acid alone, energy source alone, or ^{12}C-aspartic acid and energy source.

If a complete mixture of amino acids, including ^{14}C-glutamic acid, is added to the incubation mixture during incorporation, the radioactivity of the protein fraction of P increased in a linear manner for 3–4 hr., and is accompanied by a parallel increase in total nitrogen of the fraction.

Incorporation of ^{14}C-glutamic acid, in the presence or absence of other amino acids, is decreased if fraction P is first treated with ribonuclease or extracted with M-NaCl. The rate of incorporation by fractions so treated is markedly increased by addition of nucleic acid preparations worked up from the soluble portion of the fragmented cells. The activating effect of such nucleic acid preparations is partly (60–85 %) abolished if they are incubated either with ribonuclease or with N-NaOH at room temperature.

A Complete Fractionation of the Cerebral Phospholipids Labelled with ^{32}P. By R. M. C. Dawson, (Betty Brookes Research Fellow). (*Department of Biochemistry, Oxford University*)

Methods have been developed for measuring the specific activities of all the known phospholipids present in small samples of tissue which have been labelled with ^{32}P. A chloroform/methanol extract of the trichloroacetic acid-precipitated phospholipids was washed twice with 0·25 M-$MgCl_2$. The lipid residue obtained on evaporation was redissolved in hot methanol, and the light precipitate obtained on cooling removed. The kephalins were absorbed on MgO, and the choline-containing phospholipids separated into lecithins and sphingomyelins by saponification.

Diphosphoinositide in the methanol-insoluble fraction was hydrolysed with 6 N-HCl and the hydrolysate passed through amberlite IR 100 resin. Inositol metadiphosphate was then separated by paper chromatography using a *tert.*-butanol/trichloroacetic acid/water solvent. Phosphatidyl serine in the lipid extract was hydrolysed with methanolic NaOH. A hydrolysis product provisionally identified as glycerylphosphorylserine was separated by treatment with amberlite I.R.C. 50 followed by two-dimensional paper chromatography in propanol/NH_3 and *tert.*-butanol/trichloroacetic acid/water. Phosphatidyl ethanolamine was catalytically hydrolysed with $HgCl_2$ and glycerylphosphorylethanolamine separated by paper chromatography in phenol/water (Norman & Dawson, 1953).

A guinea-pig brain homogenate incubated with labelled phosphate under conditions which ensured a maximal incorporation into the lipid fraction (Dawson, 1953) showed a high turnover of the kephalin fraction and low turnover of the choline-containing phospholipids. Expressed as a percentage of the specific activity of the whole phospholipid fraction: methanol-insoluble 350–560 %, MgO absorbed 195 %, lecithin 9 %, sphingomyelin < 10 %. Further fractionation of the kephalins revealed a high specific activity for diphosphoinositide (950 %) while the specific activities of phosphatidyl serine and ethanolamine were below 5 %. When a labelled lipid extract was passed through amberlite IRA 400 formate resin (Kennedy, 1953) a large part of the radioactivity was removed, but the active fraction was not easily eluted with ethanolic HCl.

When slices of brain and liver were incubated with labelled phosphate, the pattern of phospholipid activities was similar to that found with homogenates. Similar results were also found with cerebral material from short term *in vivo* experiments.

REFERENCES

Dawson, R. M. C. (1953). *Biochem. J.* **53**, viii.
Kennedy, E. P. (1953). *J. Biol. chem.* **201**, 399.
Norman, J. & Dawson, R. M. C. (1953). In the Press.

Studies with a Strain of *Bacterium coli* requiring Citrulline and a Pyrimidine for Growth. By K. J. C. Back and D. D. Woods. (*Department of Biochemistry, University of Oxford*)

The mutant of *Bact. coli* (R 185–482) used in this work was isolated by Roepke (quoted by Tatum, 1946) and kindly supplied to us by Dr B. D. Davis; it requires citrulline (or arginine) and uracil (or cytosine) for growth. Similar mutants of *Bact. coli* (Davis, personal communication) and *Pseudomonas aeruginosa* (Loutit, 1952) have been reported. Reversions have always been to growth without either substance (Tatum, 1946, and present work) so that the double requirement is probably due to a mutation at a single locus. Citrulline and pyrimidine may therefore have a common precursor or, as suggested by Tatum (1946), there may be inhibitory action between chemically similar intermediates in two distinct synthetic pathways.

Uracil (10^{-4} M) was required for half-maximal growth and could be replaced by carbamyl-L-aspartate (ureidosuccinate) at the same concentration, though the lag phase was increased from 7 to 20 hr. After a single subcultivation on carbamylaspartate however the growth response curves were identical. The increased lag phase returned after a further subcultivation on uracil. This could mean that an adaptive enzyme is required for the conversion of carbamylaspartate to a true intermediate (possibly a riboside) in pyrimidine synthesis. Alternatively, as suggested by Vogel & Davis (1952) in an analogous case, it may be that carbamylaspartate is itself an intermediate but is normally metabolized by an adaptive enzyme. Carbamylaspartate is a possible precursor of pyrimidine for *Lactobacillus bulgaricus* O 9 (Wright *et al.*, 1950).

Growth on carbamyl-L-aspartate (either before or after adaptation) was inhibited by the higher

homologue carbamyl-L-glutamate when the ratio of the concentration of carbamylglutamate to carbamylaspartate was 15 to 1 or greater, and the range of carbamylaspartate concentration was $10^{-4}-5\times10^{-3}$M. After prolonged incubation (>48 hr.) growth occurred with the higher concentrations of carbamylglutamate; this was not due to reversion but apparently to the development of resistance. Growth on uracil was not affected even by the highest concentration of carbamylglutamate. The latter did not replace citrulline for growth.

If a carbamylglutamate derivative has a similar function in citrulline synthesis in bacteria to that found in animal tissues (Grisolia, 1951), and if the present mutant were found to accumulate carbamylglutamate or a derivative, the origin of the double requirement would be satisfactorily explained along the lines suggested by Tatum (1946). These points are being investigated.

REFERENCES

Grisolia, S. (1951). *Phosphorus Metabolism*, **1**, 619. Baltimore: Johns Hopkins Press.

Loutit, J. S. (1952). *Aust. J. exp. Biol. med. Sci.* **30**, 287.

Tatum, E. L. (1946). *Cold Spr. Harb. Symp. quant. Biol.* **11**, 278.

Vogel, H. J. & Davis, B. D. (1952). *Fed. Proc.* **11**, 485.

Wright, L. D., Valentik, K. A., Spicer, D. S., Huff, J. W. & Skeggs, H. R. (1950). *Proc. Soc. exp. Biol., N.Y.*, **75**, 293.

The Diazo Reacting Pigments of Serum, Urine and Bile. By P. G. Cole, G. H. Lathe and B. H. Billing. (*The Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, London, W. 6 and Postgraduate Medical School of London, W. 12*)

Cole & Lathe (1953) have separated serum bile pigments into those reacting directly and indirectly in the van den Bergh reaction. They suggested that the former consisted of two components. These have now been separated on reverse-phase partition columns of silicone-treated kieselguhr, using the system prepared from 50 vol. *n*-butanol, 45 vol. water, and 5 vol. of 0·1 M pH 6 phosphate buffer. With 6 g. of silicone-treated kieselguhr, and 3 ml. of non-polar phase, the less polar component (pigment I) has an R value of 0·3 and the more polar (pigment II) a value of 0·95. The latter pigment can be separated from interfering substances on a reverse-phase chromatogram using the two phases prepared from 17 g. ammonium sulphate, 62·5 ml. of 0·1 M pH 6 phosphate buffer, and 25·9 ml. acetone.

The visible and ultraviolet absorption spectra of these two pigments have been studied as they are eluted from the columns. In the butanol-water-pH 6 system pigment I has a flat peak at 452 mμ. and is associated with a variable amount of absorption in the ultraviolet, frequently giving a small peak in the region of 260–280 mμ. Under the same conditions pigment II has a plateau at 420–450 mμ., and absorbs in the ultraviolet. Bilirubin dissolved in the polar phase of the butanol-water-pH 6 system has a peak at 448–452 mμ. The pigments are thus very similar, but distinct from bilirubin.

Examination of a small series of sera from cases of hepatitis and obstructive jaundice shows that the relative amounts of pigments I and II are variable from patient to patient. Bile and urine from cases of obstructive jaundice have been shown to contain small amounts of bilirubin, and other diazo positive pigments in addition to pigments I and II.

REFERENCE

Cole, P. G. and Lathe, G. H. (1953). *J. clin. Path.* **6**, 99.

Action of Deoxycorticosterone Acetate and Cortisone on Response of Adrenalectomized Rats to Hypotonic Load. By D. F. Cole.* (*Department of Medicine, King's College, University of Durham, Newcastle upon Tyne*)

From measurements of the retention of sodium and chloride after giving a hypotonic saline load to rats it has been possible to calculate the change of the amount of sodium situated outside the chloride space. This is regarded as indicating changes of sodium present in the cells (Cole, 1953). In addition, the renal activity towards NaCl after hypotonic loading has been investigated in normal and adrenalectomized rats and in adrenalectomized rats treated with cortisone and DCA.

Intact animals excrete considerably more water than sodium during the first 5 hr. after loading, with an increase of retention concentration (Wolf, 1950). The diuretic response which is absent in adrenalecto-

* I.C.I. Research Fellow.

mized animals, is not restored by DCA. Treatment with cortisone and cortisone + DCA increases the water excretion after loading but the ability to reabsorb Na is less than in normals.

Adrenalectomized animals show significant losses of cell sodium after loading, in contrast to controls. Treatment with DCA only partly abolishes this but animals treated with cortisone and cortisone + DCA do not show losses of cell sodium.

It is suggested that the loss of cell sodium and accumulation of potassium after adrenalectomy are due to two underlying causes as doses of DCA sufficient to reduce cell potassium to normal do not prevent loss of sodium (Cole, 1952). Treatment with cortisone + DCA cannot truly be said to restore the ability to produce water diuresis because urine of high sodium concentration is produced and the retention concentration is not increased.

REFERENCES

Cole, D. F. (1952). *Acta Endocrinologica*, **11**, 9.
Cole, D. F. (1953). *J. Physiol.* (in the Press).
Wolf, A. V. (1950). *The Urinary Function of the Kidney*. New York: Grune and Stratton.

DEMONSTRATION

Continuous Electrophoresis on Paper. By E. S. Holdsworth. (*National Institute for Research in Dairying, University of Reading*)

The technique of continuous electrophoresis was described by Svensson & Brattsen (1949) but Grassmann & Hannig (1950) were the first to show that filter paper could be successfully used as the porous medium for supporting the electrolyte. More recently Durrum (1951) has published details of a similar type of apparatus and discussed its use in separating amino acids and proteins. The apparatus to be demonstrated is an improved form, capable of long periods of operation and was made in the author's laboratory with only ordinary workshop facilities. Substances having mobility on paper using the conventional one-dimensional electrophoresis apparatus, can be separated on a preparative scale.

A sheet of filter paper 18 × 22 in. carries a background electrolyte by gravity to the extremity of the paper where a series of drip points leads into twenty-nine collection tubes. The electrical field is applied at right angles to the gravitational flow. The mixture to be separated is introduced by a wick 1 mm. wide at some convenient point at the top of the filter paper and is separated in a manner reminiscent of mass spectroscopy. Working conditions can be varied to deal with a wide range of mobilities. Separation depends mainly on two factors (i) rate of flow down the paper, which is governed by the choice of paper and the composition of the electrolyte, (ii) rate of migration in the electrical field which can be altered by changing the pH and/or the ionic strength of the electrolyte.

The method has been used here for the separation of amino-acid mixtures, for the purification of coenzyme A and flavin-adenine dinucleotide, and, using borate buffers (Consden & Stanier, 1952), for the isolation of sugars from mixtures. The apparatus was constructed primarily for the separation of protein mixtures, e.g. by 5 days' continuous operation, 2·5 g. of freeze-dried sow's whey proteins were separated into ionophoretically homogeneous fractions and the vitamin-B_{12} 'binding' substance (cf. Gregory and Holdsworth this meeting) present in minute amounts in milk, was obtained free from the major protein constituents. Good resolution of the proteins in serum can be obtained.

REFERENCES

Consden, R. & Stanier, W. M., (1952). *Biochem. J.* **51**, xix.
Durrum, E. L. (1951). *J. Amer. chem. Soc.* **73**, 4875.
Grassmann, W. & Hannig, K. (1950). *Naturwissenschaften*, **37**, 397.
Svensson, H. & Brattsen. I. (1949). *Ark. Kemi*, **1**, 401.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **320th Meeting** *of the Biochemical Society was held in the Department of Biochemistry, Bute Medical Buildings, St Andrews, Fife, on Friday,* **17th July, 1953,** *at* 11 *a.m., when the following papers were read:*

COMMUNICATIONS

Evidence for the Structure of Ovolecithin Derived from a Study of the Action of Lecithinase C.

By C. Long and M. F. Maguire. (*Department of Biological Chemistry, University of Aberdeen*)

Both acid and alkaline hydrolysis of phosphoglycerides is accompanied by migration of the phosphate group. Nevertheless, by comparing the proportions of β-, D-α- and L-α-glycerophosphates formed on alkaline hydrolysis of naturally occurring phosphoglycerides and of synthetic phosphoglycerides of known structure, evidence was presented to show that the natural phosphoglycerides belonged to the L-α series (Long & Maguire, 1953). It has been shown by Baer & Kates (1950) that alkaline hydrolysis of synthetic phosphatidic acids is not accompanied by phosphate migration. Consequently, the conversion of a phosphoglyceride into the corresponding phosphatidic acid and alkaline hydrolysis of the latter should give glycerophosphate of configuration identical with that of the parent phosphoglyceride.

An emulsion of purified ovolecithin in 0·15M trishydroxymethylaminomethane buffer, pH 7·8, was incubated for 36 hr. at 37° with *Clostridium welchii* type A α-toxin, as source of lecithinase C. The diglyceride formed was catalytically hydrogenated in presence of Adams's catalyst. Treatment with diphenylphosphoryl chloride, followed by hydrogenolysis, gave the corresponding phosphatidic acid. The latter was hydrolysed in alkali and the glycerophosphate formed was isolated as the barium salt. Analysis showed it to be pure barium L-α-glycerophosphate, so that the ovolecithin used as starting material must also have had the L-α configuration.

During the course of this work, it has been found that only lecithins containing unsaturated fatty acids are substrates for lecithinase C. Synthetic lecithins containing only saturated fatty acids and naturally occurring lecithins after catalytic hydrogenation were not acted upon.

REFERENCES

Baer, E. & Kates, M. (1950). *J. biol. Chem.* **185,** 615.
Long, C. & Maguire, M. F. (1953). *Biochem. J.* **54,** 612.

The Enzymic Phosphorylation of D-Ribose.

By Kathleen Heald and C. Long. (*Department of Biological Chemistry, University of Aberdeen*)

Escherichia coli was grown on a solid medium with ribose as the carbon source. The washed cells (4 mg. dry wt./ml.) readily utilized ribose when incubated at 38° in medium containing only ribose and buffer, pH 7·6. Cupric ions were inhibitory at low concentrations. Sonic disintegration of the cells in 0·1M-trishydroxymethylaminomethane buffer, pH 7·6, and centrifugation yielded an almost clear cell-free supernatant which utilized ribose only when supplemented with adenosine triphosphate (0·005M) and magnesium ions (0·005M). Fluoride ions up to 0·05M were without effect.

Ribose phosphorylation was then carried out on a larger scale with the cell-free enzyme system. The adenine nucleotides were afterwards removed by mercury precipitation and the excess mercuric ion was removed as HgS. The filtrate gave a slight 'Ba-insoluble' fraction at pH 8·3, which was discarded; addition of ethanol then gave a 'Ba-soluble alcohol-insoluble' fraction which, after three further reprecipitations, was dried and analysed. It proved to be the Ba salt of a ribose phosphate.

Acid stability, rate of colour development with the orcinol reagent, paper chromatography, periodate oxidation and comparison with synthetic materials, suggest that the substance isolated is in fact the Ba salt of ribose 5-phosphate.

Dissimilation of Citric Acid by Extracts of *Aerobacter aerogenes*. By S. DAGLEY and E. A. DAWES. (*Departments of Biochemistry, Universities of Leeds and Glasgow*)

Aerobacter aerogenes can utilize citric acid as sole source of carbon for growth, and from an analysis of fermentation liquors Deffner & Franke (1939) and Brewer & Werkman (1939) concluded that citrate is dissimilated anaerobically to oxaloacetic and acetic acids as initial products. Dagley & Dawes (1953) confirmed earlier observations on the adaptive nature of the dissimilating enzyme and showed that cells have maximum activity when harvested from anaerobic media containing amounts of citrate limiting for growth.

Cell-free extracts containing the active enzyme were prepared by crushing cell paste in a Hughes (1951) press. The extract had little action on pyruvic and produced no keto-acids from malic, fumaric or succinic acids, but strong oxaloacetic decarboxylase activity was exhibited. Evolution of CO_2 from citrate was linear in the initial 30 min. and initial rates were directly proportional to the amount of extract present. Varying citrate concentration with a fixed quantity of extract revealed that approximately 1 mole of pyruvate was produced from each mole of citrate up to a citrate concentration of 6×10^{-4} M.

The rate of total keto acid and of pyruvic acid production was inhibited 83% by 0·1 M-NaF, and this suggested that Mg^{2+} was a co-factor in citrate dissimilation in addition to its known role in enzymic decarboxylation of oxaloacetate. Activity was considerably reduced by dialysis against water and stimulation could be achieved by addition of Mg^{2+} but not by Mn^{2+}. The keto acids produced were shown, by paper chromatography of the 2:4-dinitrophenylhydrazones, to be pyruvic and oxaloacetic; the latter was only detectable in ageing extracts with diminished oxaloacetic decarboxylase activity, but could be readily identified in the presence of 0·04 M-DL-malic acid as inhibitor of the decarboxylase. These observations accord with the reactions:

(1) citrate → oxaloacetate + acetate,
(2) oxaloacetate → pyruvate + CO_2.

Estimations of acetic acid and of CO_2 showed approximately 1 mole of each formed per mole of pyruvate produced, as required by reactions (1) and (2).

Reaction (1) is an apparent reversal of citrate synthesis by condensing enzyme. This view is not supported by our results which indicate that the enzyme is adaptive and suppressed by aerated growth. Furthermore, no coenzyme A dependence could be demonstrated.

REFERENCES

Brewer, C. R. & Werkman, C. H. (1939). *Enzymologia*, **6**, 273.
Dagley, S. & Dawes, E. A. (1953). *J. Bact.* (in the Press).
Deffner, M. & Franke, W. (1939). *Liebigs Ann.* **541**, 85.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.

The Mechanism of the Effect of Iron and Cobalt on the Production of Toxin by *Corynebacterium diphtheriae*. By W. A. and G. D. CLARKE. (*Wright-Fleming Institute, St Mary's Hospital Medical School, London, W.* 2)

An increase in the iron content of the medium causes a decrease in the toxin produced by cultures of *Corynebacterium diphtheriae*. Pappenheimer (1947) has shown that, for every 4 atoms of iron added to the medium (above that optimal for toxin production) 4 molecules of porphyrin and 1 molecule of toxin disappear from the culture filtrate. Pappenheimer & Hendee (1947) have shown that the cytochrome *b* content of the bacterial cells increases on increasing the iron content of the medium and suggest that diphtheria toxin is the protein moiety of diphtherial cytochrome *b*.

The effect of iron is reinvestigated using the strain (G/12/6) of the organism and modified semi-synthetic medium used for routine toxin production in this laboratory. It is found that for every 2 atoms of iron added 2 molecules of coproporphyrin and 1 molecule of toxin disappear from the culture filtrate. Between iron concentrations of 0·1 and 0·9 μg./ml. only 50% of the iron is present in the washed bacteria. The protohaemin extractable from the cells (Rawlinson & Hale, 1949) accounts for only 6% of that present in the cells.

Cobalt has an effect similar to that of iron, 2 μg./ml. causing a 60% decrease in the toxin titre with only a slight decrease in growth. Only a small percentage of the Co^{2+} is found in the washed cells and, if the metal content of the cells is compared,

Co^{2+} is more active than iron in depressing toxin production. The two metals have an equal effect on coproporphyrin formation. A growth inhibition caused by concentrations of Co^{2+} sufficient almost to inhibit the formation of toxin, is associated with a similar decrease in the haemin content of the culture. If the Fe^{2+} content of the medium is increased, the growth inhibition is prevented, though the haemin content of the cells is decreased.

It is suggested that diphtheria toxin may be unrelated to cytochrome *b* and that growth inhibition caused in *C. diphtheriae* by Co^{2+} is due to decreased formation of respiratory enzymes which, at low iron concentrations, is the growth-controlling factor.

REFERENCES

Pappenheimer, A. M. jun. (1947). *J. biol. Chem.* **167**, 251.
Pappenheimer, A. M. jun. & Hendee, E. D. (1947). *J. biol. Chem.* **171**, 701.
Rawlinson, W. A. & Hale, J. H. (1949). *Biochem. J.* **45**, 247.

The Nature of the Inhibition of Yeast Fermentation by D-Glucosone. By J. H. JOHNSTONE and I. L. S. MITCHELL. (*Department of Biochemistry, Bute Medical Buildings, St Andrews*)

In the report (Mitchell & Bayne, 1952) that D-glucosone inhibited the fermentation of glucose by baker's yeast it was suggested that the inhibition might be competitive; however, the application of the tests described by Ackermann & Potter (1949) has shown that in this process a slowly dissociating enzyme-glucosone complex is formed.

D-Glucosone inhibits the phosphorylation of glucose, fructose, mannose and glucosamine in a cold-treated yeast preparation (Krebs, Gurin & Eggleston, 1952). There is little evidence of phosphorylation of the osone in this system.

A partially purified yeast hexokinase (Kunitz & McDonald, 1946) phosphorylates D-glucosone readily while L-glucosone is unaffected. Recently, Cramer & Woodward (1952) have described the phosphorylation of D-2-deoxyglucose by a cell-free yeast extract, while Brown (1951) and Grant & Long (1952), following the earlier observations of Harpur & Quastel (1949) on the glucosamine inhibition of phosphorylation in brain tissue, have shown that glucosamine is phosphorylated by yeast hexokinase.

REFERENCES

Ackermann, W. W. & Potter, V. R. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 1.
Brown, D. H. (1951). *Biochim. biophys. Acta*, **7**, 847.
Cramer, F. B. & Woodward, G. E. (1952). *J. Franklin Inst.* **253**, 354.
Grant, P. T. & Long, C. (1952). *Biochem. J.* **50**, xx.
Harpur, R. P. & Quastel, J. H. (1949). *Nature, Lond.*, **164**, 693.
Krebs, H. A., Gurin, S. & Eggleston, L. V. (1952). *Biochem. J.* **51**, 627.
Kunitz, M. & McDonald, M. R. (1946). *J. gen. Physiol.* **29**, 273.
Mitchell, I. L. S. & Bayne, S. (1952). *Biochem. J.* **50**, xxvii.

The Significance of Urinary *p*-Hydroxyphenylacetic acid (PHPA). By K. ATERMAN, R. J. BOSCOTT and W. T. COOKE. (*Departments of Anatomy and Medicine, University of Birmingham*)

Urines from experimental animals, normal humans and hospitalized individuals were hydrolysed, fractionated and analysed by paper chromatography according to methods described elsewhere (Boscott, 1952*a*, *b*, *c*).

In contrast to normal animals, scorbutic guinea-pigs excreted in the urine (fraction I, Boscott, 1952*a*) PHPA as well as smaller amounts of *p*-hydroxyphenyllactic acid (PHPLA). Hydrolysis of the urine is not essential for the detection of these compounds although they are then found in smaller amounts; neither is it necessary to administer supplements of tyrosine. Treatment of the animals with ascorbic acid—100 mg./day given orally—caused a prompt disappearance of PHPA and PHPLA from the urine, whereas the urinary excretion of *p*-hydroxybenzoic acid and some fifteen other phenolic acids was not significantly altered. Tyrosine administration to scorbutic guinea pigs caused a rise in the urinary excretion of PHPA and PHPLA.

The influence of vitamin B_{12}, folic acid, folinic acid, cortisone, androgens, oestrogens and thyroxine on the excretion of PHPA was studied.

In the urines of twenty-three normal humans some twenty fluorescent non-phenolic and thirty-one phenolic acids have been detected in fraction I. PHPA was found in only three of these individuals; their ascorbic acid intake appeared to be low since PHPA was absent after the administration of supplements of the vitamin.

PHPA has been detected in the urine of patients with thyrotoxicosis, liver disease, pink disease, rheumatoid arthritis, Addison's disease, steatorrhoea and steatorrhoea with refractory megaloblastic anaemia. Clinical and biochemical evidence suggests the possibility that there may be a relationship between the abnormally high requirements of ascorbic acid by patients with concomitant steatorrhoea and megaloblastic anaemia and the resistance of the anaemia to treatment with vitamin B_{12}, folic acid or iron therapy, but without additional ascorbic acid.

It is suggested that in humans and guinea pigs, the urinary excretion of PHPA may be regarded as an early indication of sub-optimal intake of ascorbic acid.

REFERENCES

Boscott, R. J. (1952*a*). *2nd Int. Congr. Biochem. Abstr.* p. 349.

Boscott, R. J. (1952*b*). *Biochem. J.* **51**, xiv.

Boscott, R. J. (1952*c*). *Chem. & Ind.* p. 472.

The Effect of Dietary Deficiencies, Aminopterin, Ascorbic Acid, Chloretone, and Antibiotics, on the Urinary Excretion of Phenolic Acids in the Rat. By R. J. Boscott and S. M. Greenberg.
(*Departments of Anatomy and Pharmacology, University of Birmingham*)

Urinary phenolic acids in the rat have been studied in relation to the ascorbic acid synthesis in the animal as influenced by factors such as chloretone, antibiotics, vitamin A deficiency and aminopterin.

Paper chromatograms (Boscott, 1952) of fraction I of the urine of rats fed a complete diet in the form of rat cake, showed strikingly similar patterns to those obtained from human subjects. The most important regular difference was the presence of *p*-hydroxyphenylpropionic acid (PHPPA) in rat urine, whereas *p*-hydroxybenzoic acid (PHBA), *p*-hydroxyphenyllactic acid (PHPLA), *p*-hydroxyphenylacetic acid (PHPA), vanillic and ferulic acids, have been identified in both rat and human urines.

The number of urinary phenolic acids excreted by rats, maintained on an apparently adequate synthetic diet moderately rich in casein, was greatly reduced. Vanillic and ferulic acids which were then no longer found, are therefore not tyrosine metabolites.

Aureomycin and phthalylsulphathiazole administration prevented the urinary excretion of PHPPA but did not significantly alter the levels of other phenolic acids found in the urine. It was therefore concluded that PHPPA arises in the gut as a product of bacterial metabolism of tyrosine or phenylalanine.

Ascorbic acid administered orally to rats fed the synthetic diet, initially caused a slight reduction in the excretion of PHPA and PHPLA, whereas PHPPA excretion was unaffected. Chloretone administration, which is known to augment greatly the urinary excretion of ascorbic acid in rats, abolished the urinary excretion of PHPA, PHPLA and PHPPA.

The phenolic acid excretion of young rats fed a synthetic diet deficient in vitamin A was studied for a period of 100 days. No significant differences in phenolic acid excretion were found in these animals in comparison with litter mates maintained on a complete synthetic diet. Similarly, aminopterin did not greatly alter phenolic acid metabolism. Folinic acid administered to two rats caused a marked reduction in the urinary excretion of PHPA and PHPLA.

These investigations suggest that vitamin A does not greatly influence ascorbic acid metabolism in the rat.

The different results obtained from the chloretone and ascorbic acid experiments suggest that the rate of turnover of ascorbic acid in the rat may be very high.

REFERENCE

Boscott, R. J. (1952). *2nd Int. Congr. Biochem. Abstr.* p. 349.

The Role of Soluble Proteins in the Elimination of Carcinogens from the Animal Body. By J. G. CHALMERS. (*Cancer Research Department, Royal Beatson Memorial Hospital, Glasgow*)

The induction of tumours in animals by 3:4-benzpyrene and carcinogens of this type is well known, and investigations have been made of the rate at which these substances disappear from the animal body after injection or painting. *In vivo* combination of benzpyrene or its metabolites with tissue constituents has been described by Doniach, Mottram & Weigert (1943), Miller (1951) and Wiest & Heidelberger (1953), and it has been suggested that such combinations play an essential part in carcinogenesis.

An examination has been made by zone electrophoresis and paper chromatography of the nature of the combination *in vitro* between 3:4-benzpyrene and the proteins of human and animal serum and the results compared with the behaviour of non-carcinogenic hydrocarbons. Electrophoretic examination has shown that both types of hydrocarbons were associated with the lipoprotein fraction of the serum, and paper chromatography that the bound hydrocarbon had a high R_F value in water and was associated with protein and lipid material. The hydrocarbon was, however, loosely bound to lipoprotein and shaking with ether at room temperature was sufficient to transfer most of the fluorescent material from the serum to the ether layer.

Ultraviolet examination of the serum protein patterns after electrophoretic separation on filter paper of human, fowl, rat and mouse sera to which benzpyrene had been added *in vitro* showed that the hydrocarbon was attached to the lipoprotein fraction. In the case of rat and mouse sera the lipoprotein was relatively unstable under the conditions used in electrophoresis.

Benzpyrene colloid was injected intravenously in rats and chickens and the serum collected 1–1½ hours later was examined spectrographically and by zone electrophoresis. The fluorescent constituent of the serum appeared to be a derivative of benzpyrene and a major fraction was associated with the albumin of the serum on electrophoresis.

REFERENCES

Doniach, I., Mottram, J. C. & Weigert, F. (1943). *Brit. J. exp. Path.* **24**, 1.

Miller, E. C. (1951). *Cancer Res.* **11**, 100.

Wiest, W. C. & Heidelberger, C. (1953). *Cancer Res.* **13**, 246, 250, 255.

The Formation of Volatile Fatty Acids in Laboratory Mixtures of Minced Crops and Water, and the Occurrence of these Acids in Field Silage. By A. J. G. BARNETT and R. E. B. DUNCAN. (*Division of Agricultural Biochemistry, Department of Biological Chemistry, University of Aberdeen*)

The work already reported by Barnett & Duncan (1952, 1953*a*, *b*, *c*) on the formation of volatile fatty acids in laboratory slurries has now been extended and completed by an examination of a number of such mixtures under various conditions of temperature, aeration and anaerobiosis. It is now clear that acetic acid is predominantly formed at an early stage in all these fermentations, but that the classical belief that butyric acid is formed only at a high pH is unfounded. This acid has been found commonly in material at a pH of less than 4·0. Propionic acid is formed in all slurries at room temperature and in those at 37°, while in material of high pH, valeric and caproic acids occur frequently.

Work on field silage, which is of necessity confined to consideration of the material as an end product, has largely confirmed the findings with laboratory mixtures. Straight chain fatty acids (C_1–C_8) have been found in the large number of samples examined and while, again, C_5–C_8 acids are common in silage of high pH (approx. 5·0) butyric and valeric acids have been found in material of a pH as low as 3·77. The only branched-chain acid which has been noted upon occasion is *iso*valeric acid.

The average percentage composition of the volatile fatty acids in field silage has been compared with the corresponding figures for rumen contents (El-Shazly, 1952), the figures for the occurrence of combined lower fatty acids in the 'oil' fraction of kale (Barnett & Duncan, 1953*a*, *c*), and the results of Raper (1905) for the formation of lower fatty acids by the fusion of lactic acid with solid KOH.

REFERENCES

Barnett, A. J. G. & Duncan, R. E. B. (1952). *Biochem. J.* **52**, xvii.
Barnett, A. J. G. & Duncan, R. E. B. (1953*a*). *Biochem. J.* **53**, xxx.
Barnett, A. J. G. & Duncan, R. E. B. (1953*b*). *J. Agric. Sci.* (in the Press).
Barnett, A. J. G. & Duncan, R .E. B. (1953*c*). *Plant and Soil*, **4**, 370.
El-Shazly, K. (1952). *Biochem. J.* **51**, 640.
Raper, H. S. (1905). *J. Physiol.* **32**, 216.

The Breakdown of Amino Acids in Fermentation Mixtures of Minced Kale and Water. By H. A. Robertson and A. J. G. Barnett. (*Division of Agricultural Biochemistry, Department of Biological Chemistry, University of Aberdeen*)

Using an apparatus which has already been described (Barnett, 1952) studies have been made using the reversed phase gas chromatographic technique of James & Martin (1952), on the formation of volatile fatty acids in minced kale/water mixtures kept for 20 weeks at room temperature. Some of these results have already been reported for slurries under anaerobic conditions, under conditions of aeration and for slurries prepared with the inclusion of a small amount of SO_2 (Barnett & Duncan, 1953*a*, *b*).

As a corollary to this work, the present investigation is concerned with the breakdown of amino acids in the slurries already described. Summarizing briefly, it may be stated that one-dimensional chromatograms, run in phenol/water (4/1, w/v), indicate very little proteolysis in any of the mixtures. A complete breakdown of the initial amino acids present in the aerated slurry occurs rapidly after about 4 weeks, while a disappearance of some of the amino acids, including threonine, aspartic and glutamic acids, is evident in the anaerobic mixture. The amino acid pattern in the SO_2 slurry remains largely unchanged. The relevant two-dimensional chromatograms (phenol/water, 4/1 w/v—butanol/acetic acid/water, 4/1/5, v/v/v) have been prepared in order to investigate further these changes, particularly the disappearance of aspartic acid, glutamic acid and threonine and the appearance of α-aminobutyric acid.

REFERENCES

Barnett, A. J. G. (1952). *J. Sci. Food Agric.* **5**, 199.
Barnett, A. J. G. & Duncan, R. E. B. (1953*a*). *Biochem. J.* **53**, xxx.
Barnett, A. J. G. & Duncan, R. E. B. (1953*b*). *Plant and Soil*, **4**, 370.
James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.

Periodate Oxidation of Glycogens. By D. J. Manners. (*Department of Chemistry, University of Edinburgh*)

In continuation of studies on the structure of glycogens, end-group assays have been carried out on several additional samples of glycogen. By potassium periodate oxidation (Bell & Manners, 1952; cf. Halsall, Hirst & Jones, 1947) unit-chain lengths of 12 ± 1 glucose radicals were found for foetal-pig liver, rabbit liver III, cat liver IV, cat liver VI, and *Mytilus edulis* IV glycogens. *Helix pomatia* II, *Mytilus edulis* V and human liver (von Gierke disease) glycogens had chain lengths of 10, 9 and approx. 6 respectively. By methylation, rabbit liver glycogen had a chain length of 12 (Bell, 1935) and *H. pomatia* II one of 11–12 (Baldwin & Bell, 1940).

End-group assays have also been made by sodium periodate oxidation for 25 hr. at 2° C. (Potter & Hassid, 1948). Measurement of the formic acid liberated, and the periodate uptake, indicate that oxidation is not complete under these conditions. The end-group values so obtained are not in good agreement with those obtained by methylation or by potassium periodate oxidation of the same samples. Oxidation of maltose under similar conditions gave 2·4–2·6 moles formic acid per mole (Atherden and Manners, 1953; cf. Potter & Hassid, 1948; and Morrison, Kuyper & Orten, 1953, who obtained 3 and 2·5 moles respectively). This difference in the oxidation of the 'model' compound may account, in part, for the discrepancy between the end-group values of glycogens obtained by Cori & Larner (1951) from periodate oxidation (Potter & Hassid method) and enzymic assays.

Prolonged periodate oxidation of glycogens, followed by acid hydrolysis, and analysis of the hydrolysate for glucose, has been used to detect 1:2 or 1:3 glycosidic linkages (Hirst, Jones & Roudier, 1948; Gibbon & Boissonnas, 1950). No significant amounts of glucose could be detected either by chromatography or by glucose oxidase in experiments with five different glycogens, and two glycogen α-dextrins; it is concluded that these glycogens contain only 1:4 and 1:6 linkages.

I wish to thank Prof. E. L. Hirst, F.R.S., and Dr D. J. Bell for their interest in this work.

REFERENCES

Atherden, L. M. & Manners, D. J. (1953). Unpublished.
Baldwin, E. & Bell, D. J. (1940). *Biochem. J.* **34**, 139.
Bell, D. J. (1935). *Biochem. J.* **29**, 2031.
Bell, D. J. & Manners, D. J. (1952). *J. chem. Soc.* p. 3641.
Cori, G. T. & Larner, J. (1951). *J. biol. Chem.* **188**, 17.
Gibbon, G. C. & Boissonnas, R. A. (1950). *Helv. chim. acta*, **33**, 1477.
Halsall, T. G., Hirst, E. L. & Jones, J. K. N. (1947). *J. chem. Soc.* p. 1399.
Hirst, E. L., Jones, J. K. N. & Roudier, A. J. (1948). *J. chem. Soc.* p. 1799.
Morrison, M., Kuyper, A. C. & Orten, J. M. (1953). *J. Amer. chem. Soc.* **75**, 1502.
Potter, A. L. & Hassid, W. Z. (1948). *J. Amer. chem. Soc.* **70**, 3488.

Inhibition of Rumen β-Glucosidase Activity by Glucono-1:5-Lactone. By J. Conchie. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

The discovery that β-glucuronidase and other glucuronide-decomposing enzymes are inhibited by saccharo-1:4-lactone (Levvy, 1952) suggested that β-glycosidases in general might be inhibited by the lactones of the corresponding aldonic acids. This possibility has been tested with a β-glucosidase preparation from the sheep's rumen. The substrate was 0·005 M *o*-nitrophenyl-β-D-glucoside in phosphate-citrate buffer, pH 5·4. β-Glucosidase activity was competitively inhibited by glucono-1:5-lactone, but not by the 1:4-lactone, nor by gluconic acid itself. K_i, the dissociation constant of the enzyme-inhibitor complex, for glucono-1:5-lactone was $9{\cdot}1 \times 10^{-5}$ M, as compared with a value of $8{\cdot}9 \times 10^{-4}$ M for K_m, the dissociation constant of the active enzyme-substrate complex, for *o*-nitrophenyl-β-D-glucoside.

According to Ezaki (1940), the β-glucosidase activity of Taka diastase is inhibited by glucono-1:4-lactone and by gluconic acid: the latter does not inhibit the β-glucosidase activity of emulsin nor the α-glucosidase and β-galactosidase activities of Taka diastase.

Some properties of the rumen β-glucosidase preparation will be described.

REFERENCES

Ezaki, S. (1940). *J. Biochem., Tokyo*, **32**, 107.
Levvy, G. A. (1952). *Biochem J.* **52**, 464

Nitrogen Metabolism in *Pisum sativum*. By T. G. Hyde. (*Department of Biochemistry, University College, Dundee, University of St Andrews*)

Green peas in pod (*Pisum sativum*) were harvested at sixteen different stages of development and the changes in nitrogen distribution, amino acids and peptides, in both the pods and the seeds studied.

The pods and seeds were exhaustively extracted, the protein in the extracts being precipitated by the addition of trichloroacetic acid (TCA) to 5% w/v. This procedure enabled the changes in the total nitrogen, extracted nitrogen, non-protein nitrogen and protein nitrogen contents at the different stages to be followed.

By means of comparative paper-partition chromatography the changes in the TCA-soluble compounds were studied, the solvent mixtures of Dent (1948), Partridge (1948), Wieland & Bauer (1951) and Morrison (1953) being used. The usual amino acids and amides present in plant extracts (Dent, Stepka & Steward, 1947; Hulme & Arthington, 1950) were found in both pods and seeds. Of these compounds glutamine, α-alanine, γ-aminobutyric acid and threonine predominated, whilst glycine, pipecolinic acid, cystine, methionine,

phenylalanine and tyrosine were present only in small quantities. The main differences in the distribution of the amino acids and amides in the pods and the seeds were as follows: (*a*) the higher asparagine content of the pods, (*b*) the very high arginine content of the seeds and (*c*) the presence of β-alanine in the seeds but apparently not in the pods. Several as yet unidentified ninhydrin-reacting substances are present and it is suggested that four of these may be baikiain, ethanolamine, putrescine and, in very small amounts, γ-methylene glutamine.

I am indebted to the Agricultural Research Council for a personal grant and a grant for expenses.

REFERENCES

Dent, C. E. (1948). *Biochem. J.* **43**, 169.
Dent, C. E., Stepka, W. & Steward, F. C. (1947). *Nature, Lond.*, **160**, 682.
Hulme, A. C. & Arthington, W. (1950). *Nature, Lond.*, **165**, 716.
Morrison, R. I. (1953). *Biochem. J.* **53**, 474.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Wieland, T. & Bauer, L. (1951). *Angew. Chem.* **63**, 511.

Enzymic 11-β-Hydroxylation of 11-Deoxycorticosterone (DOC). By A. C. Brownie, J. K. Grant and W. Taylor. (*Biochemistry Department, University of Edinburgh*)

Various workers (Hayano & Dorfman, 1953; Kahnt & Wettstein, 1951; Sweat, 1951) have studied the 11-β-hydroxylation of steroids by adrenal preparations, and have demonstrated a requirement for fumarate or certain other members of the tricarboxylic acid cycle (Krebs's cycle) as 'activators' of hydroxylation. Satisfactory evidence for the mechanism of this activation is lacking.

The 11-β-hydroxylation of DOC has now been studied, using washed mitochondria prepared in sucrose from fresh beef adrenal-cortical tissue, incubated aerobically in phosphate buffer (pH 7·4) with Mg^{2+}. DOC was determined by absorption at 240 mμ, following recovery from incubation mixtures in 95 ± 3% yield, by solvent partition procedures. No DOC metabolism occurred in the absence of Krebs's cycle acids. Pyruvate was without effect. Other Krebs's cycle acids (0·002 M) activated hydroxylation. 0·0005 M-ATP enhanced this effect, but was without effect itself. In presence of ATP, α-ketoglutarate, succinate and fumarate were most effective, hydroxylating 0·9–1·0 μmole DOC/mg. mitochondria total N in 1 hr. at 37°. l-Malate, oxaloacetate and citrate were less effective. The product appeared to be almost entirely corticosterone as judged by total steroid recovery and comparison with authentic corticosterone run on paper chromatograms with various solvent systems.

Fumarate failed to activate DOC hydroxylation under anaerobic conditions. Aerobically, increasing succinate concentrations increased DOC hydroxylation, supporting the view that the reaction fumarate → succinate is not involved, and that fumarate does not function as a hydrogen acceptor.

Observations of O_2 consumption indicated that DOC hydroxylation is related to the rate of oxidation of Krebs's cycle acids present, but these studies are complicated by inhibition of respiration by the DOC.

2:4-Dinitrophenol, widely employed as an uncoupler of phosphorylation and oxidation (Loomis, & Lipmann, 1948; Cross, Taggart, Covo & Green, 1949), used in concentrations which did not inhibit oxidation of Krebs's cycle acids, caused marked inhibition of DOC hydroxylation.

It thus appears highly probable that the role of Krebs's cycle acids is to support oxidative phosphorylation for the 'sparking' of the steroid to be hydroxylated (cf. Lehninger, 1945*a*, *b*). Fumarate is not specifically involved in this role, nor does it appear to be an essential hydrogen acceptor.

REFERENCES

Cross, R. J., Taggart, J. V., Covo, G. A. & Green, D. E. (1949). *J. biol. Chem.* **177**, 655.
Hayano, M. & Dorfman, R. I. (1953). *J. biol. Chem.* **201**, 175.
Kahnt, F. W. & Wettstein, A. (1951). *Helv. chim. acta*, **34**, 1790.
Lehninger, A. L. (1945*a*). *J. biol. Chem.* **157**, 363.
Lehninger, A. L. (1945*b*). *J. biol. Chem.* **161**, 437.
Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
Sweat, M. L. (1951). *J. Amer. chem. Soc.* **73**, 4056.

The Effect of Certain Reducing Agents on the Anti-thrombic Action of Heparin. By J. GREEN. (*North West Group Laboratory, London, N.W. 3*)

Reducing agents like Na_2SO_3, $Na_2S_2O_4$, ascorbic acid, thioglycollic acid, etc., retard the clotting by thrombin of plasma and fibrinogen solutions, particularly the latter. Under certain conditions in the final clotting mixture, selective retardation of the clotting of heparinized plasma can be shown. On the other hand, when thrombin solutions are reduced, e.g. by 0·02M-thioglycollate, and inhibitory effects in the clotting mixture minimized, the anti-thrombic effect of heparin is greatly diminished, and the usual stoicheiometric relationship between added heparin and amount of thrombin bound, no longer obtains. So far, only thioglycollate, dithionite and thiol-benzoic acid have proved effective in this respect; BAL is itself oxidized in thrombin solutions.

The findings suggest that heparin normally combines with the oxidized form of some component of the thrombin complex.

Observations on the Chemical Nature of Castle's Intrinsic Factor. By A. L. LATNER and E. MCEVOY-BOWE. (*Section of Chemical Pathology, Department of Pathology, King's College, Newcastle upon Tyne*)

Using techniques involving preparative paper-strip electrophoresis (Latner, Ungley, Cox, McEvoy-Bowe & Raine, 1953), active material has been isolated from pig gastric mucosa and human gastric juice. Intrinsic factor activity has been demonstrated by studying the faecal excretion of radioactive vitamin B_{12} after oral administration to patients with pernicious anaemia (Welch & Nichol, 1952).

The pig material contains 10% N. Acid hydrolysis followed by paper chromatography demonstrated the presence of the amino acids alanine, arginine, aspartic acid, cysteine (or cystine), glutamic acid, glycine, leucine, methionine, proline, threonine and valine. There was possibly a small amount of phenylalanine. Histidine, lysine and tyrosine could not be detected. After alkaline hydrolysis, the Folin-Ciocalteu reagent indicated the presence of tryptophan. Acid hydrolysis followed by paper chromatography has also demonstrated the presence of fucose, galactose, and hexosamine (probably glucosamine and chondrosamine) as well as an unidentified spot. No uronic acid spot could be demonstrated.

The human material contained the same amino-acids and carbohydrates. In each case the hexosamine content (Smithies, 1953) was approximately 6% (expressed as glucosamine).

It would appear therefore from the analytical data available that Castle's intrinsic factor is mucoprotein in nature.

We should like to thank Dr F. T. Farmer and Dr C. C. Ungley for carrying out the clinical tests involving radioactive vitamin B_{12}. Our work is supported by a grant from the Medical Research Council.

REFERENCES

Latner, A. L., Ungley, C. C., Cox, E. V., McEvoy-Bowe, E. & Laureen Raine (1953). *Brit. med. J.* **1**, 467.
Smithies, W. R. (1953). *Biochem. J.* **53**, xxix.
Welch, A. D. & Nichol, C. A. (1952). *Ann. Rev. Biochem.* **21**, 633.

Observations on the Oxidation of Glucose and Acetate by Lactating Sheep Mammary Tissue *in vitro*. By W. G. DUNCOMBE and R. F. GLASCOCK. (*National Institute for Research in Dairying, University of Reading*)

Half-gram batches of slices of lactating sheep mammary tissue were incubated in Krebs's bicarbonate-Ringer (gas phase = 95% O_2, 5% CO_2) with sodium acetate (0·02M) and glucose (0·3 g./100 ml.) as substrates both separately and together. By labelling one substrate or the other with ^{14}C the total amount of substrate carbon oxidized after 3 hr. could be determined by displacing all the CO_2 into a high vacuum apparatus, measuring it and counting portions in a gas counter. $CH_3{}^{14}COONa$ and ^{14}C-glucose prepared from photosynthetic starch were used.

It was found that the 0·5 g. batches of slices oxidized from 0·11 to 0·39 mg. acetate carboxyl carbon within the 3 hr. when acetate was the only substrate and from 0·20 to 0·41 mg. glucose carbon

when glucose (assumed to be uniformly labelled) was the only substrate.

This first result is in accord with the findings of Folley & French (1950), but the second is not. Folley & French deduced from respiratory data that lactating ruminant mammary tissue actively oxidizes acetate as sole substrate but, unlike lactating rat mammary tissue, is quite inert to glucose. It is therefore of particular interest to find now that ruminant tissue does in fact oxidize glucose approximately as actively as it does acetate.

It was further found that the sheep slices oxidized more acetate carboxyl carbon to CO_2 in the presence of glucose than in its absence, again confirming the respiratory results of Folley & French (1950). But it was also found that they oxidized more glucose in the presence of acetate than in its absence. This was unexpected and has not been reported before for any tissue. Thus each substrate is oxidized to a greater extent in the presence of the other than in its absence.

Measurements were also made on slices metabolizing these substrates in the presence of insulin but no effect of the hormone could be detected, thus confirming the results of Balmain & Folley (1951), using respiratory methods, and of Balmain, Folley & Glascock (1953), using isotope incorporation methods.

The authors are glad to acknowledge the advice and encouragement of Dr S. J. Folley, F.R.S., in this work and to thank Mr B. W. E. Peaple for technical assistance. Thanks are also due to Dr Helen Porter of the Institute of Plant Physiology for making the radioactive starch.

REFERENCES

Balmain, J. H. & Folley, S. J. (1951). *Biochem. J.* **49**, 663.

Balmain, J. H., Folley, S. J. & Glascock, R. F. (1953). *Biochem. J.* **53**, xxvi.

Folley, S. J. & French, T. H. (1950). *Biochem. J.* **46**, 465.

The Metabolism of Nitrophenols by Certain Bacteria. By J. R. Simpson and W. C. Evans.
(*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

From the filter beds of a biological detoxication plant, two organisms* (*Pseudomonas* spp.) were isolated which decomposed *ortho*-nitrophenol (strain SO) and *para*-nitrophenol (strain SP) respectively. Each strain utilized the appropriate nitrophenol as its sole source of organic carbon, but had no action on the other isomer.

Under aerobic conditions, in a liquid medium containing 100 p.p.m. of nitrophenol with inorganic salts, maximum growth occurred at pH 7·0–7·5 for SO, and 7·5–8·0 for SP. In this medium, one gram-ion of nitrite was produced per mole of nitrophenol decomposed. Under anaerobic conditions, however, no nitrite production was found.

After growth on *p*-nitrophenol medium, SP showed simultaneous adaptation to quinol but not to catechol. SO was adapted to catechol, after growth on *o*-nitrophenol medium, and not to quinol. The adaptation of both organisms to phenol was very rapid and the correct interpretation is not clear. *Cis-cis*-muconic acid was rapidly converted to β-ketoadipic acid by freeze-dried SO cells grown in *o*-nitrophenol medium. Similarly treated SP grown in *p*-nitrophenol medium did not bring about this conversion. The chromatographic evidence for phenolic intermediates will be discussed.

o- and *p*-Aminophenols were found not to be intermediates in the metabolic pathway by adaptation criteria, and by absence of nitrite and ammonia production. Similarly, the presence of ammonium ion or hydroxylamine in the cell suspension had no influence on nitrite formation. Hence, there is no evidence for a primary reduction of the nitro group, followed by elimination of ammonia or hydroxylamine and reoxidation of either to nitrite.

These facts support the view that the mechanism of detoxication is an oxidative elimination of the nitro group, before fission of the aromatic ring. The catechol thus formed by SO is then utilized according to the pathway of Evans, Smith, Linstead & Elvidge (1951) through *cis-cis*-muconic and β-ketoadipic acids.

An organism was isolated from soil which utilized 2:4-dinitrophenol producing nitrite ions under conditions similar to the above. The perfusion of chloramphenicol medium through soil also caused nitrite accumulation.

* We wish to thank Monsanto Chemicals Ltd., Ruabon, for these cultures.

REFERENCE

Evans, W. C., Smith, B. S. W., Linstead, R. P. & Elvidge, J. A. (1951). *Nature, Lond.*, **168**, 772.

Formation of Oligosaccharides during the Enzymic Hydrolysis of β-Glucosides. By E. M. Crook and B. A. Stone.* (*Department of Biochemistry, University College, London*)

Many disaccharide-hydrolysing enzyme preparations have been shown to transfer glucose, fructose and galactose residues from maltose, sucrose and lactose to other sugars, liberating glucose and forming higher oligosaccharides (Pazur & French, 1952; Pan, Nicholson & Kolachov, 1953; Blanchard & Albon, 1950; Bealing & Bacon, 1953; Aronson, 1952; Roberts & McFarren, 1953; Pazur, 1952, 1953).

A similar reaction has now been demonstrated with the β-glucoside cellobiose, using a partially purified enzyme preparation from *Aspergillus niger* (Rhom & Haas, Enzyme 19) which has strong cellulase and cellobiase activity. Cellobiose solutions (7·5% w/v) were incubated in pH 5·0 acetate buffer at 40° and the reaction followed by paper chromatography using downward multiple development in butanol:pyridine:water (Jeanes, Wise & Dimler, 1951). The sugars were detected with the benzidine-trichloroacetic acid reagent of Bacon & Edelman (1951). Cellobiose moves more slowly than glucose. Spots moving more slowly than cellobiose appear during the first few hours and disappear during the later stages of the reaction leaving glucose as the sole end product. Between cellobiose and glucose a spot of comparatively low intensity appears and disappears. Of the upper spots, that immediately above cellobiose is the last to disappear.

A culture filtrate from the mould *Myrothecium verrucaria*, an extract of a bran culture of *Aspergillus aureus* (Norman Evans & Rais, Manchester) and a preparation from the gut of the snail *Helix pomatia* give an identical series of spots when incubated with cellobiose under the above conditions.

Substituting low molecular weight cellodextrins for cellobiose gives rise to a similar series of spots with Enzyme 19.

* Officer, Defence Standards Laboratories, Commonwealth Department of Supply, Australia.

REFERENCES

Aronson, M. (1952). *Arch. Biochem. Biophys.* **39**, 370.
Bacon, J. S. D. & Edelman, J. (1951). *Biochem. J.* **48**, 114.
Bealing, F. J. & Bacon, J. S. D. (1953). *Biochem. J.* **53**, 277.
Blanchard, P. H. & Albon, N. (1950). *Arch. Biochem.* **29**, 220.
Jeanes, A., Wise, C. S. & Dimler, R. (1951). *Analyt. Chem.* p. 415.
Pan, S. C., Nicholson, L. W. & Kolachov, P. (1953). *Arch. Biochem. Biophys.* **42**, 406.
Pazur, J. H. (1952). *J. biol. Chem.* **199**, 217.
Pazur, J. H. (1953). *Science*, **117**, 356.
Pazur, J. H. & French, D. (1952). *J. biol. Chem.* **196**, 265.
Roberts, H. R. & McFarren, E. F. (1953). *Arch. Biochem.* **43**, 233.

Separation of Cerebrospinal Fluid Proteins by Paper Electrophoresis. By J. C. Eaton and Mary D. Gardner. (*Department of Biochemistry, Glasgow Royal Infirmary*)

For paper electrophoresis approximately 2·5 mg. of protein is desirable. Owing to the low concentration of protein in cerebrospinal fluid it is necessary to concentrate the fluid 50–100 times to enable this amount of protein to be applied to the paper strip.

To concentrate the protein, Kabat, Moore & Landow (1942) dialysed under pressure, Esser, Heinzler & Wild (1952) dialysed *in vacuo* and Bücher, Matzelt & Pette (1952) precipitated the proteins by Komplexon. These procedures are slow and involve the risk of bacterial decomposition and presumably for this reason all these authors found it necessary to maintain the fluid at a low temperature. Owing to the high proportion of salts to protein in cerebrospinal fluid, evaporation alone cannot be employed since the high concentration of salts and pH changes precipitate the proteins.

By passing the fluid through a column of Amberlite Monobed MB 1 ion exchange resin it has been possible to remove salts without denaturation of protein, the close association of anion and kation exchangers in this resin minimizing the pH changes. The fluid thus obtained was then freeze-dried and redissolved in a minimal quantity of saline and the proteins separated by electrophoresis on a paper strip in the usual way.

The cerebrospinal fluid was found to contain the same electrophoretic protein fractions as occurred in the serum though not necessarily in similar proportions. In the samples examined there was no evidence of the additional fractions described by Kabat *et al.* and by Bücher *et al.*

REFERENCES

Bücher, Th., Matzelt, D. & Pette, D. (1952). *Klin. Wschr.* **30**, 325.

Esser, H., Heinzler, F. & Wild, H. (1952). *Klin. Wschr.* **30**, 228.

Kabat, E. A., Moore, D. H. & Landow, H. (1942). *J. clin. Invest.* **21**, 571.

Isomerism of 2:4-Dinitrophenylhydrazones of Monobasic α-Oxo Acids. By H. B. Stewart.
(*Department of Biochemistry, University of Cambridge*)

Paper chromatography of the 2:4-dinitrophenylhydrazone of pyruvic acid yields two spots (Seligson & Shapiro, 1952; Hockenhull, Hunter & Herbert, 1953). The 2:4-dinitrophenylhydrazones of glyoxylic and α-ketobutyric acids are now found also to yield two spots in paper chromatography.

The two isomers were separable on columns of bentonite and cellulose with ether-methanol mixtures (cf. Braddock *et al.* 1953). Infrared and ultraviolet spectra show the absence of bands specific for C=O and imino groups in one isomer (A), for which there is also evidence of restricted dissociation of the carboxyl group except in strong alkali. This indicates a strong intramolecular hydrogen bond between the imino group of the hydrazine linkage and the C=O of the carboxyl group in A. The addition of excess NaOH (about 2 equiv.) to B rapidly produces a characteristic red-brown colour, while similar treatment of A produces no such effect, though in a great excess of NaOH, A is slowly converted into a highly coloured red-brown substance.

On this evidence A and B are believed to be *cis* and *trans* isomers (respectively) around the C=N band of the hydrazone, the *cis* isomer being stabilized by a hydrogen bond between the imino and carboxyl groups.

Paper chromatography of the α-methyl-α-2:4-dinitrophenylhydrazone of glyoxylic acid in which the imino group is no longer present results in the formation of only one spot. *p*-Nitrophenylhydrazones of the monobasic α-oxo acids yield two spots and it seems probable that hydrogen bonding between the imino and *o*-nitro-groups in the *trans* form is not a prerequisite for the stabilization of the geometric isomers.

My thanks are due to Dr R. N. Haszeldine of the Department of Chemistry for the preparation and interpretation of the infrared spectra.

REFERENCES

Braddock, L. I., Garlow, K. Y., Grim, L. I., Kirkpatrick, A. F., Pease, S. W., Pollard, A. J., Price, E. F., Reissman, T. L., Rose, H. A. & Willard, M. L. (1953). *Analyt. Chem.* **25**, 301.

Hockenhull, D. J. D., Hunter, G. D. & Herbert, M. W. (1953). *Chem. & Ind. (Rev.)*, 127.

Seligson, D. & Shapiro, B. (1952). *Analyt. Chem.* **24**, 754.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **321st Meeting** *of the Biochemical Society was held in the Department of Biochemistry, The School of Medicine, Leeds* 2, *on Friday, September* 18*th*, 1953, *at* 11 *a.m., when the following papers were read:*

COMMUNICATIONS

Oxidative Phosphorylation in Insect Sarcosomes. By S. E. Lewis and E. C. Slater. (*Molteno Institute, University of Cambridge*)

Sarcosomes in the flight muscles of insects resemble mammalian heart-muscle sarcosomes in their morphology (see Cleland & Slater (1953) for review), their high concentration of cytochrome (Keilin, 1925; Levenbook, 1952) and their high respiratory activity (Watanabe & Williams, 1951). However, attempts to link this respiratory activity with the synthesis of adenosine triphosphate (ATP) have up to now been unsuccessful (see Sacktor, 1953).

Using the procedure previously described (Slater, 1953), oxidative phosphorylation has now been demonstrated during the oxidation of α-ketoglutarate by sarcosomes obtained from the flight muscles of the blowfly *Calliphora erythrocephala* (2–6 days old). After removing the head and abdomen, the flies were briefly ground without sand in a mortar with 0·20M-sucrose, 0·002M-ethylenediaminetetraacetic acid (Versene) and the sarcosomes separated by differential centrifugation. In a typical experiment with 2-day-old flies, sarcosomes containing 1·2 mg. protein consumed 2·01 μatoms oxygen and 1·08 μmoles α-ketoglutarate, and 2·07 μmoles of phosphorus were esterified (P : O = 1·03). Under the same conditions, with the same preparation, the P : O ratio with succinate as substrate was 0·14. The P : O ratio for the oxidation of α-ketoglutarate to succinate is, therefore, $\{2{\cdot}07 - 0{\cdot}14\ (2{\cdot}01 - 1{\cdot}08)\}/1{\cdot}08 = 1{\cdot}80$. This is lower than the value of 3 obtained for heart-muscle sarcosomes (Slater & Holton, 1953). The low ratio is not due to high ATP-ase activity, since sufficient hexokinase was added to compete with the ATP-ase completely. Neither is it due to phosphomonoesterase activity, which is very small in these sarcosomes. It cannot yet be stated whether the low ratio is a property of insect sarcosomes *in vivo*, or is due to losses of phosphorylating enzymes during the isolation procedure.

S. E. L. is on leave from D.S.I.R. Pest Infestation Laboratory, London Rd., Slough. E. C. S. is working on behalf of the Agricultural Research Council.

REFERENCES

Cleland, K. W. & Slater, E. C. (1953). *Quart. J. micr. Sci.* (in the Press).
Keilin, D. (1925). *Proc. Roy. Soc.* B, **98**, 312.
Levenbook, L. (1952). Unpublished observations quoted by D. Gilmour in *Insect Physiology*, p. 404, ed. by Roeder, K. D. London: Chapman and Hall.
Sacktor, B. (1953). *J. gen. Physiol.* **36**, 371.
Slater, E. C. (1953). *Biochem. J.* **53**, 521.
Slater, E. C. & Holton, F. A. (1953). *Biochem. J.* (in the Press).
Watanabe, M. I. & Williams, C. M. (1951). *J. gen. Physiol.* **34**, 675.

The Effect of Dinitrophenol on Insect Sarcosomes. By E. C. Slater and S. E. Lewis. (*Molteno Institute, University of Cambridge*)

The stimulation of the respiration of mitochondria by the addition of either dinitrophenol or adenosinetriphosphate (ATP)-utilizing systems such as hexokinase and glucose is now well known. However, with liver mitochondria (Lardy & Wellman, 1952) or heart-muscle sarcosomes (Slater, unpublished) these two effects are not additive, i.e. the addition of dinitrophenol to mitochondria already supplied with excess hexokinase and glucose has little effect on the respiration. In the case of insect sarcosomes, however, the addition of 10^{-4} M-dinitrophenol increased the rate of oxidation of α-ketoglutarate 3·2-fold, even in the presence of excess hexokinase and glucose. This concentration of dinitrophenol decreased the P : O ratio from 1·03 to 0·62 (P : α-ketoglutarate, 0·83), but the increase of respiration more than compensated for the lower ratio, with the unusual result that the rate of synthesis of esterified phosphorus was increased by 36%. This is a consequence of three factors: (1) the substrate-linked phosphorylation with α-ketoglutarate is little affected by dinitrophenol (Judah, 1951; Hunter, 1951); (2) the dinitrophenol-sensitive respiratory chain phosphorylation contributes a relatively small

proportion of the total phosphorylation in the insect-muscle sarcosomes; (3) the rate of respiration is limited by some process which is affected by dinitrophenol.

These findings are understandable on the basis of the following description of respiratory chain phosphorylation (cf. Hunter, 1951; Lardy & Wellman, 1953):

$$(1)\ AH_2+B+C \rightarrow A\sim C+BH_2;$$
$$(2a)\ A\sim C+ADP+H_3PO_4 \rightleftharpoons A+C+ATP;$$
$$(2b)\ A\sim C+H_2O \rightarrow A+C,$$

where A and B are adjacent hydrogen-carriers in the respiratory chain and C is a substance or grouping essential for their interraction. The rate of respiration is governed by the regeneration of A by reactions (2a) and (2b). Reaction (2a) is not rate-limiting in heart sarcosomes which give a high P : O ratio, but is slow in the poorly phosphorylating insect sarcosomes. Reaction (2b) is normally relatively slow, but is accelerated by dinitrophenol. The reverse reaction (2a) is not operative in our experiments, because ATP is rapidly removed by the hexokinase-glucose.

The finding that the rate of synthesis of ATP can be increased by the addition of a typical uncoupling agent, even when the ATP is being rapidly utilized, is some support of Lardy's (1952) theory of the mode of action of certain hormones.

E. C. S. is working on behalf of the Agricultural Research Council. S. E. L. is on leave from D.S.I.R. Pest Infestation Laboratory, London Road, Slough.

REFERENCES

Hunter, F. E. (1951). *Phosphorus Metabolism*, **1**, 297, ed. by W. D. McElroy and Bentley Glass. Baltimore: The Johns Hopkins Press.
Judah, J. D. (1951). *Biochem. J.* **49**, 27.
Lardy, H. A. (1952). *The Biology of Phosphorus*, p. 131, ed. by Lester F. Wolterink. Michigan State College Press.
Lardy, H. A. & Wellman, H. (1952). *J. biol. Chem.* **195**, 215.
Lardy, H. A. & Wellman, H. (1953). *J. biol. Chem.* **201**, 357.

A Study of the Complexes of Some Simple Peptides with Zinc and Cadmium Ions in Aqueous Solution. By D. J. Perkins. (Introduced by N. H. Martin.) (*Department of Chemical Pathology, St George's Hospital Medical School, London, S.W.* 1)

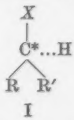
The zinc and cadmium complexes of a number of glycyl dipeptides and some other simple peptides have been examined by a potentiometric method. The effect of peptide structure on the stability constants of the metal complexes has been studied. The presence of peptide bonds was found to decrease these constants when compared with those of the constituent amino acids. The relative effects of the structure of each amino acid in a series of glycyl peptides was found to be similar to those for the free amino acids.

The Molecular Rotations of Triterpenoid Alcohols and their Derivatives. By W. Klyne (*Postgraduate Medical School, London, W.* 12) and W. M. Stokes (*Providence College, Providence, R.I., U.S.A.*)

Stokes & Bergmann (1952) discussed the rotation contributions of hydroxyl and related groups in steroids, and tried to apply to these compounds the empirical rules of Marker (1936) regarding acyclic compounds. Mills (1953) showed by reference to monoterpenoids that the application of Marker's rules to cyclic compounds is unjustified. The work of Mills (1952, 1953) on optical rotations and of Dauben, Dickel, Jeger & Prelog (1953) on asymmetric synthesis has shown that the steroid and glyceraldehyde conventions for formulae are in agreement. Klyne (1952) had previously correlated the triterpenoids with the steroids.

The method of rotational analysis used by Stokes & Bergmann (1952) can now be applied generally to cyclic alcohols in the light of the work of Mills and others cited above. The principles are as follows. An asymmetric centre C* in a *cyclo*hexane ring system may be represented in perspective as in (I), where X is a substituent and R, R′ are the ring-

members adjacent to C* together with their substituents; the hydrogen atom (H) is on that side of C* farthest from the observer.



I

If *X* is a hydroxyl, acetoxyl or benzoyloxy group, the magnitude and sign of the rotation contribution of C* is dependent on the degree of alkyl substitution in R and R′.

(i) If R and R′ are both $-CH_2.C.$, the rotation contribution of C* is small and attempts to predict its sign are unwise.

(ii) If R is $-CH_2.C.$, and R′ is $-\dot{C}.C$ or $-\dot{C}.C.$, the rotation contribution of C* is negative (and often large).

(iii) If R is $-\dot{C}.C$ or $-\dot{C}.C$ and R′ is $-CH_2.C.$, the rotation contribution of C* is positive (and often large). The contributions of acetoxyl and benzoyloxy groups are generally larger than those of the corresponding hydroxyl groups. These rules probably apply also to *cyclo*pentane rings.

The results for triterpenoids are with few exceptions consistent within themselves and with other evidence regarding the stereochemistry of the triterpenoids.

REFERENCES

Dauben, W. G., Dickel, D. F., Jeger, O. & Prelog, V. (1953). *Helv. chim. acta*, **36**, 325.

Klyne, W. (1952). *J. chem. Soc.* p. 2916.

Marker, R. E. (1936). *J. Amer. chem. Soc.* **58**, 976.

Mills, J. A. (1952). *J. chem. Soc.* p. 4976.

Mills, J. A. (1953). *Chem. & Ind.* p. 218.

Stokes, W. M. & Bergmann, W. (1952). *J. org. Chem.* **17**, 1194.

Synthesis and Oxidation of Citric Acid in Mammary Gland Homogenates. By C. Terner.
(*National Institute for Research in Dairying, University of Reading*)

Evidence presented previously (Terner, 1951) supports the view that the citric acid cycle is operating in the mammary gland. Moore & Nelson (1952), using a cylophorase preparation of lactating guinea pig mammary gland, found that added citrate was oxidized only slowly if at all and concluded that 'not only does mammary tissue not oxidize citrate, but it contains an inhibitor to this oxidative step of the tricarboxylic acid cycle'.

Since citric acid is an intermediate of the Krebs cycle, the inability of the mammary gland to oxidize citrate at adequate rates would rule out the cycle as a major metabolic pathway in this tissue.

In the present work whole homogenates of lactating mammary gland of the guinea pig were found capable of synthesizing citrate from pyruvate or acetate in the presence of fumarate and of oxidizing added citrate at rapid rates. Pyruvate was removed more rapidly than added citrate and the amount of citrate accumulating during the metabolism of pyruvate was approximately equivalent to the difference between the amounts of pyruvate and of added citrate disappearing. In the presence of fluoroacetate (see Liebecq & Peters, 1949), fumarate and pyruvate, when present in equivalent amounts, were almost quantitatively converted to citrate, while either fumarate or pyruvate alone gave rise to only small amounts of citrate.

The homogenates had a considerable endogenous respiration which was not greatly increased by the addition of pyruvate, acetate or citrate, although these substances could be shown by analytical methods to be actively metabolized. *p*-Nitrophenol (*p*NP) which had little effect on the endogenous respiration or on the respiration in the presence of fumarate alone, caused a large increase of respiration in the presence of fumarate plus pyruvate or acetate, and of citrate or α-ketoglutarate, without correspondingly accelerating the rate of disappearance of these substrates. Thus, *p*NP increased the ratio O_2 consumed/substrate oxidized to a value approaching the theoretical ratio for the complete oxidation of the substrate. According to these findings various substrates appear to be oxidized incompletely in mammary homogenates. The observation of incomplete oxidation made by analytical

and respiratory measurements aided by the use of nitrophenols has been taken as evidence suggestive of the utilization of the substrates in synthetic reactions (Terner, 1951; Melrose & Terner, 1953). In the presence of fluoroacetate the amounts of oxygen taken up, pyruvate disappearing and citrate accumulating were approximately equivalent and were increased by *p*NP to the same extent.

REFERENCES

Liebecq, C. & Peters, R. A. (1949). *Biochim. Biophys. Acta*, **3**, 215.
Melrose, D. R. & Terner, C. (1953). *Biochem. J.* **53**, 296.
Moore, R. O. & Nelson, W. L. (1952). *Arch. Biochem. Biophys.* **36**, 178.
Terner, C. (1951). *Biochem. J.* **50**, 145.

The Enzyme Complement of Bean Root Cells. By Edith Robinson. (*Department of Botany, Leeds University*)

The bean root is suitable experimental material for the study of metabolic changes which occur during the growth of the plant cell. Serial sections cut from the apex of the root towards the base contain cells in progressively more advanced stages of development ranging from the meristematic to the mature state. Quantitative measurements made with such sections can be reduced to a 'per cell' basis by dividing the section values by the number of cells each contains. Estimations of growth, protein content, invertase, dipeptidase, phosphatase, glycine oxidase and proteolytic enzyme activity have been made. The activity of all enzymes changes during the growth of the cell. Invertase, phosphatase, dipeptidase and glycine oxidase show maximum activity, and protein content is highest when the cell reaches its maximum length. All these quantities decrease after growth ceases. Activity of proteolytic enzymes continues to increase in cells which finished growth. It is tentatively suggested that the level of proteolytic enzymes may determine the decrease in activity of the other enzymes studied.

The Separation of α-Keto Acids in Blood and Urine by Paper Chromatography. By M. J. H. Smith and K. W. Taylor. (*Department of Chemical Pathology, King's College Hospital Medical School, Denmark Hill, London, S.E.* 5)

1:2-Diamino-4-nitrobenzene (DANB) reacts with α-keto acids to yield stable derivatives which may be separated by paper chromatography (Hockenhull & Floodgate, 1952; Hockenhull, Hunter & Herbert, 1953). A satisfactory separation of the DANB derivatives of pyruvic, α-ketoglutaric, oxaloacetic, phenylpyruvic and α-ketobutyric acids has been achieved using Whatman no. 4 paper and a solvent mixture consisting of ethanol, amyl alcohol and 0·880 ammonia solution. The derivatives form yellow spots which show a deep yellow fluorescence in ultraviolet light. The R_F values show slight variation from day to day, due probably to minor changes in the solvent mixture and environmental temperature. The ranges of R_F values found for the DANB derivatives are 0·10–0·15 for oxaloacetic acid, 0·24–0·35 for α-ketoglutaric acid, 0·55–0·66 for pyruvic acid, 0·68–0·74 for α-ketobutyric acid and 0·78–0·85 for phenylpyruvic acid.

DANB has been used for the detection of α-keto acids in blood and urine. The reagent in acid solution is added to the urine or to the filtrate from blood which has been deproteinized by metaphosphoric acid. The derivatives are extracted with ethyl acetate and then removed from the organic solvent with caustic soda solution. After acidification of the caustic soda solution the derivatives are re-extracted with ethyl acetate which is evaporated to dryness and the residue dissolved in ethanol, the ethanolic solution being applied to the paper for chromatography.

Pyruvic and α-ketoglutaric acids were always found in blood and urine samples from normal subjects and diabetic patients, but oxaloacetic acid was never detected (cf. El Hawary & Thompson, 1953). A spot with an R_F value of between 0·86 and 0·93 was seen in about half of the chromatograms, but has not yet been identified. Phenylpyruvic acid was found in the urine of two cases of phenylpyruvic oligophrenia. Pyruvic and α-ketoglutaric acids only were detected in the urine of normal rats.

The quantitative estimation of the α-keto acids in blood and urine is being studied by elution of the spots with ethanol and measurement of the optical densities of the resulting solutions at 280 mμ.

REFERENCES

El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.
Hockenhull, D. J. D. & Floodgate, G. D. (1952). *Biochem. J.* **52**, 38.
Hockenhull, D. J. D., Hunter, G. D. & Herbert, M. W. (1953). *Chem. & Ind. (Rev.)*, p. 127.

Effect of Inhibitors on the Metabolism of *Microsporum*. By A. J. E. Barlow, F. W. Chattaway and C. C. Thompson. (*Department of Dermatology, Huddersfield Royal Infirmary, Department of Biochemistry, School of Medicine, Leeds*)

Endogenous respiration of *Microsporon canis* has been studied by Melton (1951) who examined the effect of various fungicidal substances on the respiration of shake cultures, and Bentley (1953) has determined the Q_{O_2} of mycelial mats. Using mycelial mats of *M. canis* and *M. audouini* grown on Sabouraud's broth at room temperature for 14–20 days it has been shown that fluoride (0·1 M), arsenite (0·01 M), iodoacetate (0·017 M) and cyanide (0·001 M) are powerful inhibitors of endogenous respiration. Fluoroacetate (0·036 M) and malonate (0·03 M) had only a slight inhibitory effect. The mycelial mats showed a respiratory quotient of 1 and Q_{O_2} (N) values were in the range 100–200.

Preformed mycelial mats of these organisms floated on to replacement medium containing phosphate buffer (0·066 M, pH 7) and potassium arsenite (0·01 M) and incubated at room temperature for 6 days lead to the accumulation in the medium of pyruvic and α-ketoglutaric acids and a third unidentified keto acid. The first two of these acids have been identified by chromatography, ultraviolet absorption spectrum and m.p. of their 2:4-dinitrophenylhydrazones and the pyruvate derivative by analysis also. The corresponding derivative of the third acid, obtained in low yield, has $\lambda_{max.}$ 355 mμ and $\lambda_{min.}$ 300 mμ in ethyl acetate. The yield of total keto acids is considerably increased by the addition of glucose (0·1 M) to the medium but not by galactose, sucrose or succinic acid and only slightly by mannose. The addition of fluoride (0·15 M) or iodoacetate (0·02 M) to the arsenite-glucose medium reduces the yield of total keto acid by 90%. Total keto-acid production in the presence of glucose (0·1 M) is of the order of 150 μg./ml. and is not greatly altered by pH, between pH 5–7; arsenite is essential for accumulation of keto acids to occur at all pH values. These findings are similar to those of Hockenhull, Wilkin & Winder (1951) who found accumulation of pyruvate and α-ketoglutarate in high yield in cultures of *Penicillium chrysogenum*, arsenite being essential at pH 7 but not at pH 5·4.

No significant differences have been found in the behaviour of *Microsporon canis* and *M. audouini* and the results provide preliminary evidence as to possible mechanisms for carbohydrate metabolism in these fungi.

REFERENCES

Bentley, M. L. (1953). *J. gen. Microbiol.* **8**, 365.
Hockenhull, D. J. D., Wilkin, G. D. & Winder, F. G. (1951). *Nature, Lond.*, **168**, 1043.
Melton, F. M. (1951). *J. invest. Dermat.* **17**, 27.

The Site of Conversion of Carotene Injected into Rats. By J. G. Bieri and C. J. Pollard (introduced by S. K. Kon). (*Department of Biochemistry and Nutrition, The University of Texas, Galveston, U.S.A.*)

When carotene solubilized in water with Tween 40 (Bieri, 1951) is injected intravenously into vitamin A-deficient rats containing only traces of vitamin A in the serum and organs high levels of vitamin A in the serum result within a few hours (Bieri & Pollard, 1953). In order to obtain information as to the possible site of conversion of the injected carotene, a series of studies was carried out with rats subjected to various surgical procedures before the injection of carotene. Deficient rats weighing 100–140 g. were operated upon under ether anaesthesia and immediately given 75–100 μg. β-carotene in aqueous solution through the tail vein. After 3–8 hr. the animals were killed and vitamin A and carotene determined in the serum, liver and kidneys.

Control rats, either intact or subjected to mock operations, produced serum vitamin A levels of 57–157 μg./100 ml. after injecting carotene. From

2·7 to 5·8 μg. vitamin A per rat were in the combined livers and kidneys. When the bile duct was ligated and severed, the vitamin A resulting from injected carotene was 93–102 μg./100 ml. serum, and 3·3–3·9 μg. in the liver and kidneys. Thus, the carotene was not transported by the bile to the intestine for conversion.

When the small intestine of rats was removed before administration of carotene, the conversion was not impaired. Vitamin A in the serum ranged from 24 to 107 μg./100 ml., and from 1·0 to 5·1 μg. were in the liver kidneys. The small intestine thus does not play a significant part in converting injected carotene.

Removal of the kidneys before carotene injection did not alter the amount of vitamin A formed, (72–161 μg./100 ml. serum, and 1·5–2·3 μg. per liver). When 60–75 % of the liver was removed and the animals injected, appreciable vitamin A was still found. The sera contained 20–76 μg./100 ml., and the remaining liver with the kidneys had 1·2–4·6 μg.

The variations in survival time and in the dosage of carotene in the different experiments, as well as the individual variation among rats, do not make it possible to state that the removal of any organ affected the conversion of carotene to vitamin A more than did the removal of another organ. It is evident that with either the small intestine or more than one-half of the liver removed, the formation of vitamin A from injected carotene is not appreciably reduced.

REFERENCES

Bieri, J. G. (1951). *J. Nutr.* **44**, 327.
Bieri, J. G. & Pollard, C. J. (1953). *Fed. Proc.* **12**, 409.

Tryptophan-Tryptophanase Reaction. Consideration of the Nature of the Enzyme-Coenzyme-Substrate Complex. By H. Gooder and Frank C. Happold. (*Department of Biochemistry, The University, Leeds*)

A study of the effect of certain structural analogues of tryptophan upon the tryptophan-tryptophanase reaction shows that:

(*a*) Indole and indolyl acids inhibited the reaction strongly while slight inhibition was given by α-amino-β-3-indenyl-propionic acid when preincubated with the enzyme.

(*b*) Indene, D-tryptophan, indolylethylamine and *N*-methyl tryptophan failed to inhibit the reaction.

The enzyme system did not produce indole with any indole containing substrate other than L-tryptophan nor was ammonia or pyruvic acid produced from α-amino-β-3-indenyl-propionic acid.

The relationship of these results to possible mechanisms of the enzyme reaction is discussed.

It is suggested that combination between L-tryptophan and the enzyme surface occurs through the ring nitrogen atom as also through the carboxyl group of the substrate. The L-tryptophan may combine with the coenzyme, pyridoxal phosphate, through the amino group of the former and the free carboxyl of the latter; the coenzyme being attached to the apo-enzyme by the phosphate group of the pyridoxal phosphate. Such a complex would provide a highly resonating network in which electron transfers could take place, and under correct energy conditions these could rupture the C_3–C_β bond in the tryptophan molecule. No intermediate would be demonstrated under these conditions since the other entity produced (in addition to indole) could be an α-amino acrylic acid-pyridoxal phosphate complex. Production of ammonia and pyruvic acid could arise from the reaction of this complex with water. Such a mechanism would account for the failure of previous workers to find an intermediate in the enzyme reaction and also offer an explanation as to why indole production is not merely a reversal of the tryptophan desmolase system.

A Possible Model of the Tryptophan-Tryptophanase Action. By J. W. Baker (introduced by F. C. Happold). (*Department of Organic Chemistry, The University, Leeds*)

There is some evidence that maximum activity in, for example, anti-bacterial (Albert, 1951) and oestrogenic (Clark, 1950; Rideal & Schulman, 1939) activity is closely associated with the 'area of flatness' of the molecule, which permits adsorption of the system on the substrate in a unimolecular layer. Such adsorption may involve functional groups (e.g. hydrogen bonding) and van der Waals forces, necessitating highly specific stereochemical requirements.

By the use of molecular models it is shown that (1) pyridoxal phosphate and tryptophan molecules fit neatly together to form a molecular complex in which the interacting aldehyde group of the former and the amino-group of the latter are brought into close juxtaposition, and (2) in this complex with L-tryptophan (but *not* with the D-form), the various groups through which, the results of Gooder & Happold (preceding abstract) suggest, bonding with the substrate occurs, all lie in a plane.

A tentative mechanism is indicated whereby decomposition of this one, common, molecular complex in three different ways could give rise, respectively, to indole fission, deamination and decarboxylation reactions.

REFERENCES

Albert, A. (1951). *Selective Toxicity*, p. 95. Methuen: London.
Clark, E. R. (1950). *J. chem. Soc.* p. 3397.
Rideal, E. K. & Schulman, J. (1939). *Nature, Lond.*, **144**, 100.

The Fate of Intravenously Injected ^{131}I-Labelled Antigens and 3:5-Diiodotyrosine in Immune and Normal Rabbits. By A. E. Almond, G. E. Francis, J. D. Hawkins and A. Wormall. (*Department of Biochemistry and Chemistry, Medical College of St Bartholomew's Hospital, London, E.C.* 1)

In earlier investigations (Francis, Hawkins & Wormall, 1953) it was shown that intravenously injected horse serum γ-globulins, trace-labelled with ^{131}I, are more rapidly removed from the blood stream of rabbits if the animals have previously received immunizing injections of horse serum γ-globulins.

Similar studies have now been made with a more foreign antigen, highly iodinated proteins (labelled with ^{131}I), and here also a significant difference is found between normal rabbits and rabbits previously immunized against iodinated proteins. The intravenously injected antigen disappeared appreciably more rapidly during the first half-hour from the blood stream of the immune rabbits than it did in the case of normal rabbits, irrespective of whether the labelled antigen was iodinated bovine serum γ-globulin (the antigen used for immunization) or iodinated rabbit serum γ-globulin. In all these experiments the difference is most marked when only small amounts of antigen are injected into the blood stream.

Experiments have also been made with the corresponding hapten, 3:5-diiodotyrosine (labelled with ^{131}I). Removal of the intravenously injected hapten from the blood stream takes place more rapidly with rabbits previously immunized with iodinated bovine serum γ-globulin, the differences in the amount remaining in the blood being in these cases most noticeable in the period 1–5 days after the injections.

Evidence has been obtained which suggests that there may be fairly rapid liberation of relatively diffusible substances containing ^{131}I from highly iodinated proteins after their injection into the blood stream. It is advisable, therefore, to limit the use of these proteins, in investigations of this type, to short-term experiments.

REFERENCE

Francis, G. E., Hawkins, J. D. & Wormall, A. (1953). *Biochem. J.* **53**, xx.

Phosphorus Metabolism in the Housefly *Musca domestica*. By F. P. W. Winteringham, Patricia M. Loveday and G. C. Hellyer. (*Department of Scientific and Industrial Research, Pest Infestation Laboratory*)

For the purpose of studying the biochemistry of insecticidal action mechanisms *in vivo* the distribution of phosphorylated intermediates in normal and in poisoned flies is being studied. Adult flies are fed for 24 hr. with aqueous dextrose containing carrier-free $H_3{}^{32}PO_4$. They are then provided with aqueous dextrose only for a further period of 24 hr., subjected to mild cyclopropane anaesthesia, and immersed in liquid nitrogen. The chilled flies are washed free of external ^{32}P-contamination. After removing the appendages the muscle-rich thoraces are homogenized and extracted in an ethanol-formic acid-water mixture at $-10°$. Some of the washed insects have been sectioned and the histological distribution of the ^{32}P determined autoradiographically by a technique developed for water-soluble tracers (Winteringham, Harrison & Hammond, 1950). The phosphorus compounds present in the muscle

extracts (about 90 % of the total ^{32}P) are resolved by ascending unidimensional paper chromatography using an acetone-formic acid solvent (Burrows, Grylls & Harrison, 1952). Chromatography proceeds in 'evacuated' all-glass columns at 0° which ensures rapid and complete saturation with the solvent vapour. The strips are dried and radiometrically scanned automatically (Winteringham, Harrison & Bridges, 1952). In this way the labelled intermediates are determined quantitatively in samples equivalent to as little as $\frac{1}{100}$ part of the thoracic muscle of one fly. Two compounds, believed to be hexose phosphates, run together in this solvent. After location by scanning they are eluted quantitatively and concentrated on to a fresh strip of paper in one operation and resolved by running in an ammonia-methanol solvent (Burrows *et al.* 1952). On the basis of R_F values, rates of hydrolysis, etc. five of the separated components behave as ATP, two hexose phosphates, phosphoglyceric acid and orthophosphate. There is little difference in the ratios of the intermediates recovered whether the flies are killed (in liquid nitrogen) while in flight or under anaesthesia. The autoradiographs have demonstrated a high concentration of labelled material in the gut wall. Its composition appears to be the same as that found in the muscle. When the flies are killed by brief immersion in hot water the inorganic-phosphate/ATP ratio is greatly increased.

This communication is made by permission of the Department of Scientific and Industrial Research.

REFERENCES

Burrows, S., Grylls, F. S. M. & Harrison, J. S. (1952). *Nature, Lond.*, **170**, 800.
Winteringham, F. P. W., Harrison, A. & Bridges, R. G. (1952). *Nucleonics*, **10**, 52.
Winteringham, F. P. W., Harrison, A. & Hammond, J. H. (1950). *Nature, Lond.*, **165**, 149.

The Stabilization of Elastin by a Polysaccharide. By G. C. Wood. (*Department of Medicine, University of Leeds*)

Elastic tissue (ox ligamentum nuchae) from which the collagen has been removed by treatment with boiling dilute acetic acid contains, interfibrillarly, a polysaccharide whose degradation products (sulphate, reducing material and amino sugar) appear when the tissue is dissolved under the action of elastase (Hall, Reed & Tunbridge, 1952). It was suggested that degradation of this polysaccharide might be essential for the dissolution of elastin. As a further indication of this, elastase has been separated into two fractions and indirect evidence suggests that one of these preferentially attacks the polysaccharide component of elastin (Hall, 1953).

Much of the polysaccharide may be extracted from collagen-free elastin by alkaline 10 % (w/v) solutions of calcium chloride and appears to be similar to chondroitin sulphuric acid (CSA) in that it contains sulphate, galactosamine and a uronic acid. It seemed of interest, therefore, to examine the action of elastase on CSA. The substrate was prepared from bovine nasal septa and the reducing material liberated when solutions were incubated with unfractionated elastase at 37° for various periods of time and over a wide pH range was estimated. There was no evidence from these experiments that the CSA was hydrolysed by the enzyme preparation.

In an attempt to obtain a measure of the cohesion of elastin, load/extension curves of strips of ox ligamentum nuchae were determined in the range 0–20 % extension in the direction of orientation of the fibrils. The curves are linear and unaffected by previous extension unless some treatment which decreases the cohesion of the material is interposed. The slope, $(df/dE)_a$, of the curves for strips from which extraneous protein and lipid have been removed and that, $(df/dE)_b$, of the curves for strips from which, in addition, collagen has been removed, were determined. Prolonged treatment with alkaline 10 % (w/v) solutions of calcium chloride at 37° have no effect on $(df/dE)_a$ or $(df/dE)_b$, although such treatment is known to remove much of the polysaccharide from finely divided elastin. Treatment with hyaluronidase at pH 5·2 has no effect on $(df/dE)_b$ but reduces $(df/dE)_a$ to a limited extent. Treatment with elastase preparations for various periods of time reduces both $(df/dE)_a$ and $(df/dE)_b$, ultimately to zero.

There is thus some doubt as to whether the polysaccharide present in elastin plays an important part in its stability. In any case it would appear to be something other than CSA.

REFERENCES

Hall, D. A. (1953). Following Abstract.
Hall, D. A., Reed, R. & Tunbridge, R. E. (1952). *Nature, Lond.*, **170**, 264.

Studies on the Complex Nature of the Elastin-Elastase System. By D. A. HALL. (*Department of Medicine, Leeds University*)

Banga in 1951 expressed doubt as to the proteolytic activity of elastase. Hall, Reed & Tunbridge (1952) further suggested that the important enzymic process is mucolytic, while according to Lansing, Rosenthal, Alex & Dempsey (1953) elastase acts by liberating lipid from a lipoprotein complex. The present author has been unable to substantiate this, and has shown, moreover (1952), that the enzyme preparations used by Banga and by Lansing were possibly complex in nature, attacking more than one component of the substrate.

Fractionation of the enzyme with ammonium sulphate caused the precipitation of the activity over a considerable range of concentrations indicating the possibility of more than one component. There was, however, little evidence that fractions at different ammonium sulphate concentrations differed other than quantitatively in their activity. Slight separation of two components was observed after chromatography with ammonium sulphate solutions, but here the properties of the proteins appeared too alike to permit the use of the method for preparative purposes (see Hall & Wewalka, 1951). Similar results were observed on chromatography with aqueous acetone.

The most successful separation of the enzyme components has been obtained by adsorption on calcium phosphate and alumina. Batch experiments furnished two fractions. The substrates for testing these fractions were collagen free elastin preparations treated with alkali for varying periods of time. The two fractions appeared to be, respectively, rich in a component specific for the alkali labile fraction of the substrate, and in a component which preferentially attacked the residue after alkali treatment.

Although conclusive proof will have to await the isolation of two pure enzymes and two pure substrates there does appear to be definite evidence in favour of a complex system in which at least part of the substrate is mucoprotein in nature and the enzyme correspondingly mucolytic.

REFERENCES

Banga, I. (1951). *Z. Vitam.- Horm.- u. Fermentforsch.* **4**, 50.
Hall, D. A. (1952). *2nd Int. Congress Biochem. Abstracts*, p. 272.
Hall, D. A., Reed, R. & Tunbridge, R. E. (1952). *Nature, Lond.*, **170**, 264.
Hall, D. A. & Wewalka, F. (1951). *Nature, Lond.*, **168**, 685.
Lansing, A. I., Rosenthal, T. B., Alex, M. & Dempsey, E. W. (1953). *Anat. Rec.* **114**, 555.

Enzymic Estimation of Citric Acid. By S. DAGLEY and E. A. DAWES. (*Department of Biochemistry, University of Leeds, and Department of Biochemistry, University of Glasgow*)

The preparation of cell-free extracts that dissimilate citrate to pyruvate, acetate and CO_2 has suggested a method for citric acid estimation. Dagley & Dawes (1953) have shown that the enzyme is strongly developed when *Aerobacter aerogenes* is cultured anaerobically in media containing citrate limiting for growth. These conditions may be obtained by growth at 37° in 10 or 5 l. flasks filled to the neck with medium of the following composition: trisodium citrate dihydrate, 9 g.; $(NH_4)_2SO_4$, 1 g.; KH_2PO_4, 2 g.; and $MgSO_4, 7H_2O$, 0·2 g. per l. When the cells had been harvested, about 10 g. cells paste were crushed in the Hughes (1951) press and taken up in phosphate buffer to provide 60 ml. of translucent extract after centrifugation. To 1 ml. of citrate solution was added 1 ml. of phosphate buffer (pH 7·0) containing 0·4 g./l. $MgSO_4.7H_2O$ and 1 ml. of extract, and after incubation at 37° for 15 min. 3 ml. of 10% (w/v) trichloracetic acid solution was added and the pyruvate formed was determined by the method of Friedemann & Haugen (1943). For freshly prepared extracts a linear relation existed between citrate concentration and absorptiometer readings up to a concentration of 6×10^{-4} M of the former in the 3 ml. of incubation mixture; at higher concentrations the reaction ceases when conversion of citrate to pyruvate is not complete. Frozen extracts retained activity on storage but the extent

of the linear range of the calibration curve was reduced after freezing and thawing. Complete recovery was obtained for citrate added to peptone broth and the following compounds did not interfere with the determination: aspartic, glutamic, succinic, malic and fumaric acids, serine, glycine, alanine and phenylalanine. The method has been used to follow disappearance of citrate from bacterial culture media.

REFERENCES

Dagley, S. & Dawes, E. A. (1953). *J. Bact.* (in the Press).
Friedemann, T. E. & Haugen, G. (1943). *J. biol. Chem.* **147**, 415.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.

Production of Keto Acids from Acetate by a Vibrio. By S. DAGLEY and M. D. PATEL. (*Department of Biochemistry, University of Leeds*)

Dagley, Dawes & Morrison (1951) harvested *Aerobacter aerogenes* from media supplying glucose and compounds of the tricarboxylic acid cycle as sole sources of carbon and aerated non-proliferating suspensions with acetate-phosphate solutions: pyruvate accumulation was only observed when the cells had previously utilized acetate for growth. We have extended these observations for a vibrio (Happold & Key, 1932) that utilizes a wide range of compounds in growth. Cells were harvested from defined media supplying the following as sole sources of carbon: citrate, succinate, malate, fumarate, mandelate, benzoate, *p*-hydroxybenzoate, phenylacetate and phenol. Suspensions of equal turbidities (0·05–0·1 mg./ml. dry wt.) were then made from each batch in growth media supplying (*a*) the original substrate, (*b*) acetate as sole carbon sources. Samples were withdrawn from (*a*) and (*b*) at suitable time intervals during growth and the cells, after separation by centrifugation, were aerated for one hour with acetate-phosphate solution. The total keto-acid concentration in the latter was then determined by the method of Friedemann & Haugen (1943). In this way it was possible to study the development of the ability of a given batch of cells to produce keto-acids from acetate. In medium (*b*) the ability was developed progressively during growth; but cells growing in (*a*) lacked this capacity in every case. The latter cells, however, were able to oxidize acetate readily in the Warburg respirometer; and further indication that keto acid excretion is to be associated primarily with growth processes rather than respiration was furnished by omitting the source of N from (*b*), when growth was precluded and development of the ability of the cells to produce keto acids from acetate was arrested.

Production of keto acids from acetate was observed for cells that utilized in growth fatty acids of chain length up to C_{10}. It may be suggested that β-oxidation occurred and that the vibrio developed a dicarboxylic acid cycle for C_2 fragments and acetate utilization. On the other hand cell-free extracts have been prepared, from cells grown on a number of carbon sources including fatty acids, that oxidize citrate with an uptake of two O per mole; whilst from chromatography the chief keto acid accumulating in acetate oxidation appears to be α-ketoglutaric.

REFERENCES

Dagley, S., Dawes, E. A. & Morrison, G. A. (1951). *J. gen. Microbiol.* **5**, 508.
Friedemann, T. E. & Haugen, G. (1943). *J. biol. Chem.* **147**, 415.
Happold, F. C. & Key, A. (1932). *J. Hyg., Camb.*, **32**, 573.

The Reactions of some Haemoglobin Derivatives with ^{131}I-labelled *p*-Iodophenylhydroxylamine and *p*-Iodonitrosobenzene. By H. JACKSON and R. THOMPSON. (*Christie Hospital and Holt Radium Institute, Manchester* 20)

p-Iodophenylhydroxylamine (IPhNHOH) and *p*-iodonitrosobenzene (IPhNO) have been shown to penetrate red cells with facility and combine firmly with the cell pigment (Crick & Jackson, 1953). Methaemoglobin is formed concurrently in the cells *in vivo* and *in vitro*, but only transiently in the former instance. The reformation of oxyhaemoglobin is not accompanied by loss of the combined iodo-compound

from the cell. With crystalline haemoglobin solutions, no initial formation of methaemoglobin has been observed.

The reactions of radioactive IPhNHOH and IPhNO with solutions of crystalline rat oxyhaemoglobin, carboxyhaemoglobin, methaemoglobin and alkaline haematin have now been examined and shown to take place in stoichiometric proportions. Phenylhydroxylamine (PhNHOH) and nitrosobenzene (PhNO) were previously known to react with oxyhaemoglobin (Heubner, Meier & Rhode, 1923; Jung, 1940). Keilin & Hartree (1943) studied the formation of the pigment complexes with haemoglobin and alkaline haematin, and concluded that reaction was due to nitrosobenzene and that phenylhydroxylamine underwent prior oxidation to this substance. Their spectroscopic examination of the action of oxygen, carbon monoxide and hydrosulphite on the pigment complexes led them to believe that the nitrosobenzene component was readily dissociated by the influence of these reagents, liberating unbound pigments. Whilst similar spectroscopic changes occur when IPhNHOH and IPhNO are used instead of PhNHOH and PhNO in the above reactions, the radioactive tracer reveals that molecular proportions of the iodo-compounds are, in fact, retained. Prior oxidation of the hydroxylamino- group to nitroso- is also not a necessary preliminary to reaction with haemoglobin pigments; for example, carboxyhaemoglobin reacts directly with IPhNHOH without change in absorption spectrum.

It is concluded that the reactions of IPhNHOH and IPhNO with various haemoglobin pigments are likely to be paralleled by PhNHOH and PhNO, and that spectroscopic data alone is inadequate in studies of these reactions.

REFERENCES

Crick, J. & Jackson, H. (1953). *Brit. J. Pharmacol.* **8**, 87.
Heubner, W., Meier, R. & Rhode, H. (1923). *Arch. exp. Path. Pharmak.* **100**, 149.
Jung, F. (1940). *Biochem. Z.* **305**, 248.
Keilin, D. & Hartree, E. F. (1943). *Nature, Lond.*, **151**, 390.

FORTHCOMING PAPERS

It is hoped to publish the following papers in the next issue of the *Biochemical Journal*:

Synthesis of L-ascorbic acid in plants and animals. By F. A. Isherwood, Y.-T. Chen and L. W. Mapson

Isolation of D-glyceric acid from cress seedlings and its relationship to the synthesis of L-ascorbic acid. By F. A. Isherwood, Y.-T. Chen and L. W. Mapson

Biological synthesis of L-ascorbic acid: the conversion of L-galactono-γ-lactone into L-ascorbic acid by plant mitochondria. By L. W. Mapson, F. A. Isherwood and Y.-T. Chen

Oxidative phosphorylation coupled with the oxidation of α-ketoglutarate by heart-muscle sarcosomes. 2. P:O ratio. By E. C. Slater and F. A. Holton

The analysis of adenosine triphosphate and adenosine diphosphate preparations by paper ionophoresis. By H. E. Wade and D. M. Morgan

The distribution of ascorbic acid between the particulate and non-particulate components of adrenal and liver cells. By P. Hagen

Biosynthesis of fatty acids by slices and cell-free suspensions of mammary gland. By G. Popják and A. Tietz

Paper-chromatographic analysis of Dakin's samples of 'β-hydroxyglutamic acid'. By C. E. Dent and Dorothy L. Fowler

Studies in the biochemistry of micro-organisms. 91. The colouring matters of *Penicillium islandicum* Sopp. Part 3. Skyrin and flavoskyrin. By B. H. Howard and H. Raistrick

Chemical action of X-rays on nucleic acids and related substances in aqueous systems. 2. The mechanism of the action of X-rays on nucleic acids in aqueous systems. By G. Scholes and J. Weiss

Organic acid metabolism of *Penicillium chrysogenum*. 1. Lactate and acetate. By D. J. D. Hockenhull, Mary Herbert, A. D. Walker, G. D. Wilkin and F. G. Winder

The fission of tobacco mosaic virus and some other nucleoproteins by strontium nitrate. By N. W. Pirie

The action of some α-amylases on amylose. By R. Bird and R. H. Hopkins

The purification of aconitase. By J. F. Morrison

Studies on the determination of the sequence of amino acids in peptides and proteins:

2. The separation of the methyl esters of *N*-2:4-dinitrophenyl derivatives of amino acids by adsorption chromatography and the free amino groups of insulin. By C. M. Fletcher, A. G. Lowther and W. S. Reith
3. The synthesis of 3-(4′-dimethylamino-3′:5′-dinitrophenyl)hydantoin derivatives of various amino acids, and their use for the determinations of *N*-terminal amino acids. By G. G. Evans and W. S. Reith
4. The synthesis of 3-(4′-dimethylamino-3′:5′-dinitrophenyl)-2-thiohydantoin derivatives of various amino acids, and their use for aminoacid-sequence determinations. By W. S. Reith and N. M. Waldron

The extent of conversion of food protein to microbial protein in the rumen of the sheep. By I. W. McDonald

Purification and properties of phosphoprotein phosphatase from ox spleen. By T. A. Sundararajan and P. S. Sarma

The synthesis of coenzyme A by *Lactobacillus arabinosus* 17–5. By W. S. Pierpoint and D. E. Hughes

The mechanism of β-amylase action:

1. 'Multichain' action on amylose. By R. H. Hopkins and B. Jelinek
2. 'Multichain' action of amylose fission products. By R. Bird and R. H. Hopkins

The formation of D-3-phosphoglyceric acid from pentoses by *Escherichia coli*. By E. D. Bergmann, U. Z. Littauer and B. E. Volcani

The utilization of non-protein nitrogen in the bovine rumen. 8. The nutritive value of the proteins of preparations of dried rumen bacteria, rumen protozoa and brewer's yeast for rats. By Mary L. McNaught, E. C. Owen, Kathleen M. Henry and S. K. Kon

Studies on the biosynthesis of blood pigments. 1. Haem synthesis in haemolysed erythrocytes of chicken blood. By E. I. B. Dresel and J. E. Falk

Synthesis of cellulose by *Acetobacter xylinum*. 1. Micromethod for the determination of celluloses. By M. Schramm and Shlomo Hestrin

Biochemical studies of toxic agents. 5. Observations on the fate of ^{35}S-labelled arylsulphuric acids following their administration to the rat. By Joyce B. Hawkins and L. Young

Levels of enzymes of the direct oxidative pathway of carbohydrate metabolism in mammalian tissues and tumours. By Gertrude E. Glock and Patricia McLean

'ROCHE' VITAMINS

	PACKINGS (*weight in grams*)		
Vitamin A			
Vit. A acetate cryst.	1·0		
Vit. A alcohol cryst.	1·0		
Vit. A palmitate cryst.	1·0		
Vitamin B-Complex			
Aneurine hydrochloride	1·0	5·0	10·0
Co-Carboxylase	0·1		
Folic acid	1·0	10·0	
Riboflavin	0·5	1·0	10·0
Nicotinic acid	5·0	10·0	25·0
Nicotinamide	5·0	10·0	25·0
Calcium *d*-pantothenate	1·0	5·0	10·0
Pyridoxine hydrochloride	0·5	1·0	5·0
d-Biotin	0·1	0·5	1·0
p-Aminobenzoic acid	1·0	5·0	10·0
Choline chloride	5·0	10·0	25·0
Inositol	5·0	10·0	25·0
Vitamin C			
l-Ascorbic acid	1·0	10·0	25·0
Vitamin E			
Racemic *a*-Tocopherol	0·5	1·0	5·0
Racemic *a*-Tocopheryl acetate	1·0	5·0	10·0
Vitamin K			
Menaphthone	1·0	5·0	10·0

Detailed Lists on Request

ROCHE PRODUCTS LTD.
WELWYN GARDEN CITY, ENGLAND

GERRARD'S STAINS & REAGENTS for MICROSCOPY

All GERRARD Stains & Reagents are prepared and standardized under the control of practical microscopists

also

Micro-Slides Coverslips
Staining Dishes Slide Boxes

and all general

EQUIPMENT & CHEMICALS

T. GERRARD & CO, LTD.
46a–48 PENTONVILLE ROAD, N.1
TELEPHONE TERminus 8006/7

THE BIOCHEMICAL JOURNAL

Decennial Index of Authors & Subjects

1937–1946 VOLUMES 31–40

EDITED BY
E. BOYLAND & R. K. CALLOW

PRICE 35*s.*
(20*s.* to members of the Biochemical Society)

Orders to
CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE
200 EUSTON ROAD, LONDON, N.W.1

BIOCHEMICAL SOCIETY SYMPOSIA 11

Biological Transformations of Starch and Cellulose

EDITED BY
R. T. WILLIAMS

Papers read at a Symposium held on 21 February 1953, by:
Stanley Peat, E. J. Bourne, W. J. Whelan, Helen Porter, G. O. Aspinall, M. V. Tracey, A. T. Phillipson, R. G. Fargher

10*s.* 6*d. net*

CAMBRIDGE UNIVERSITY PRESS

E. K. BOWMAN LTD.

Established 1889

61, 61A & 63 HOLMES ROAD, KENTISH TOWN
LONDON, N.W. 5
Telephone: Gulliver 1653

★

SPECIALISTS IN ALL TYPES OF
ANIMAL CAGES.
PELLET & CUBE TYPE FOOD HOPPERS & BASKETS.
CONSTANT LEVEL WATER TROUGHS.
C/PLATED ON BRASS SCREWED CAPS & NOZZLES
FOR PLASMA BOTTLES
ALSO 8 & 10 oz. FLATS

★

SEND FOR ILLUSTRATED CATALOGUE

'WELLCOME'

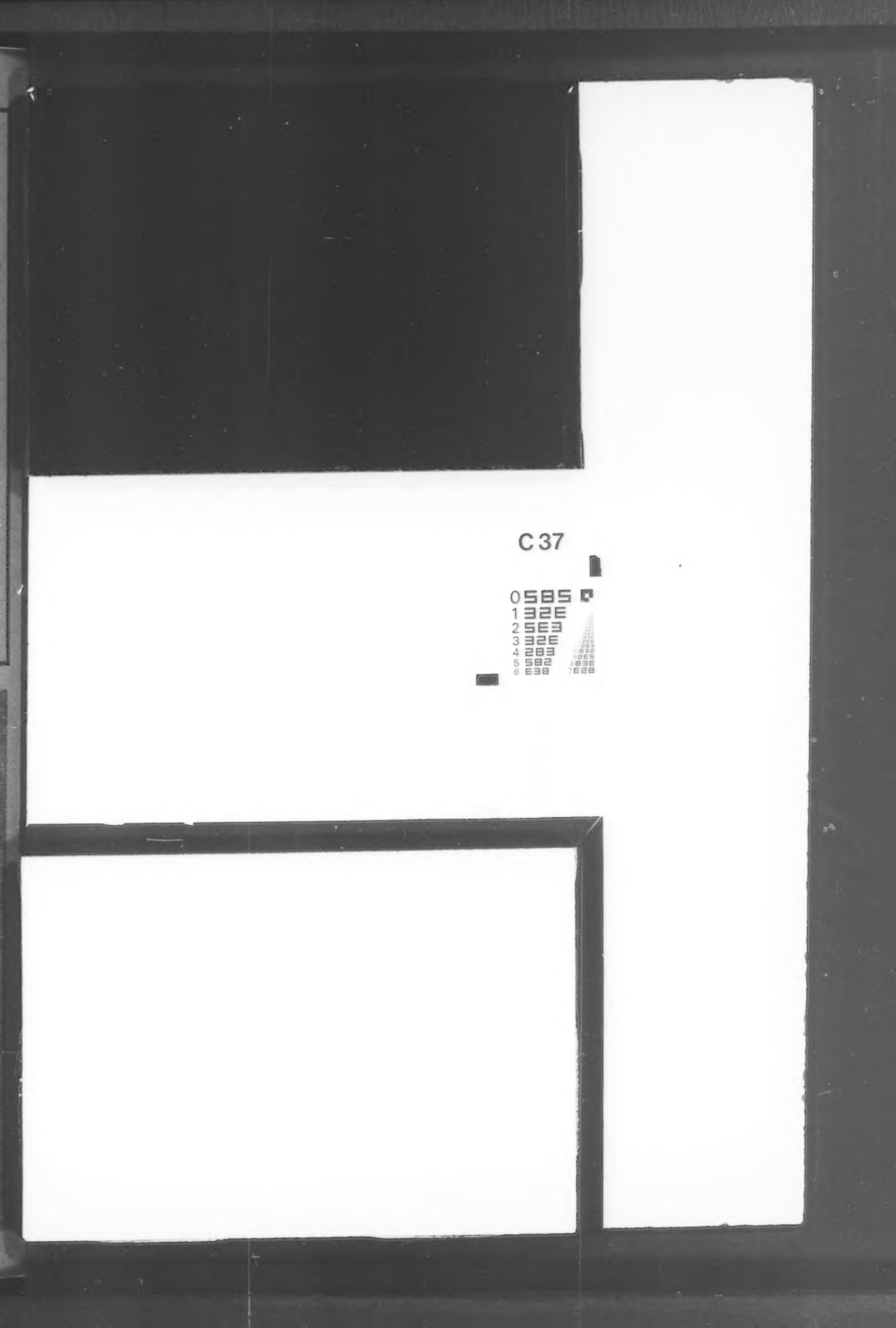
DIAGNOSTIC AND LABORATORY MATERIALS

● AGGLUTINABLE SUSPENSIONS ● AGGLUTINATING AND PRECIPITATING SERA
● CULTURE MEDIA ● OLD TUBERCULIN ◇ OPACITY TUBES ● PENICILLINASE
● REAGENTS FOR COMPLEMENT FIXATION AND FLOCCULATION TESTS
● THE 'AGLA' BRAND MICROMETER SYRINGE
● SERA FOR IDENTIFICATION OF ANAEROBES

Details and Price List on application

Obtainable from: THE WELLCOME RESEARCH LABORATORIES
Langley Court, Beckenham, Kent. Telephone: Beckenham 3422 or from:
BURROUGHS WELLCOME & CO. *(The Wellcome Foundation Ltd.)* LONDON

Printed in Great Britain at the University Press, Cambridge


C37